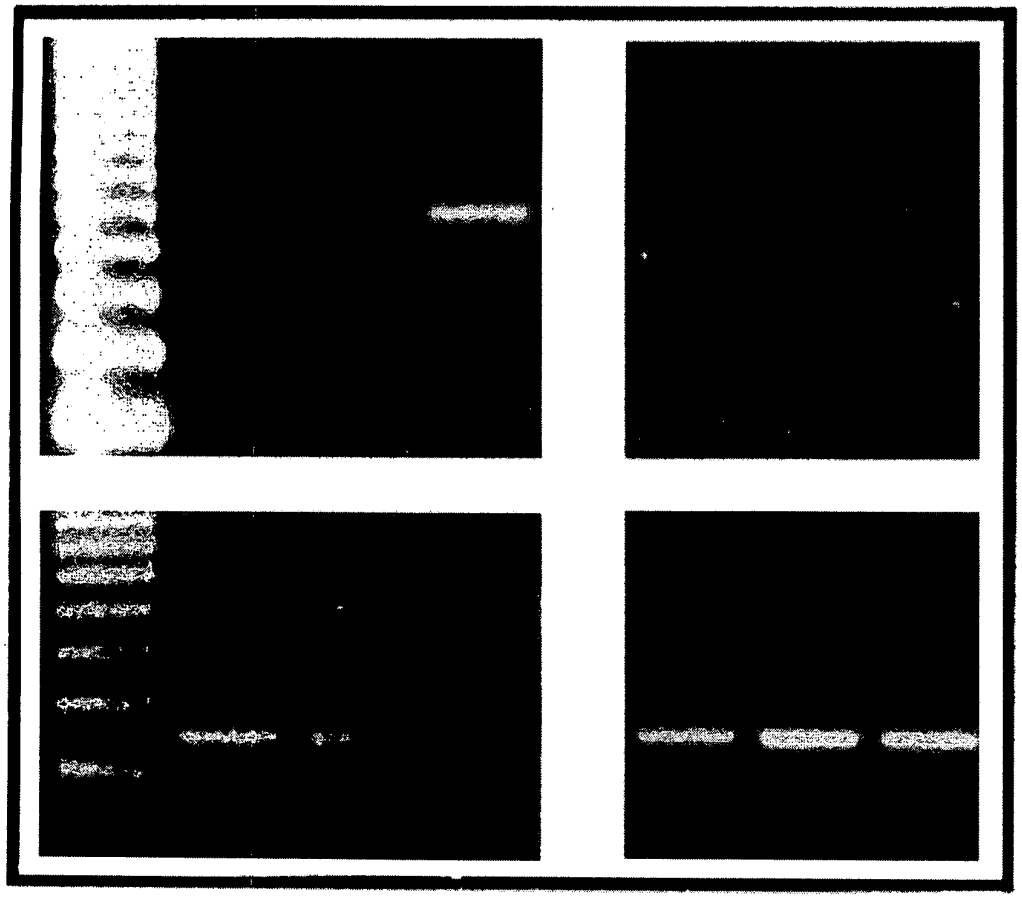
EXHIBIT A

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo

prior to following CsA administratior



TF

GAPDH

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Exhibit A

EXHIBIT B

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo

Form PTO-1449 Substitute	U.S. Department of Commerce Patent and Trademark Office	Application Number	10/037,341
		Filing Date	January 4, 2002
INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)		First Named Inventor	David Baltimore et al.
		Art Unit	1636
		Examiner Name	D. Guzo
		Attorney Docket No.	75723-ZA/JPW/GJG

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.) date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	1	Declaration of Dr. Inder Verma; and	
	2	Second Declaration of Dr. Inder Verma.	

EXAMINER SIGNATURE	DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609: Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1]Applicant's unique citation designation number (optional). [2]Applicant is to place a checkmark here if English language Translation is attached.

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Exhibit G

Docket No. 74753/JPW/GJG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

A Merged Proceeding of *Ex Parte* Reexamination Control Nos:

90/007,503 and 90/007,828
Filed April 4, 2005 Filed December 2, 2005

Merged Pursuant to May 4, 2006 Merger Decision
Group Art Unit: 3991 Examiner: B.M. Celsa

Patentees: David Baltimore, Ranjan Sen, Phillip A. Sharp, Harinder Singh, Louis Staudt, Jonathan H. Lebowitz, Albert S. Baldwin Jr., Roger G. Clerc, Lynn M. Corcoran, Patrick A. Baeuerle, Michael J. Lenardo, Chen-Ming Fan, and Thomas P. Maniatis

Patent No.: 6,410,516 B1 Serial No: 08/464,364

Issue Date: June 25, 2002 Filed: June 5, 1995

For : NUCLEAR FACTORS ASSOCIATED WITH TRANSCRIPTIONAL REGULATION

1185 Avenue of the Americas
New York, New York 10036

Mail Stop *Ex Parte* Reexamination
Central Reexamination Unit
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. INDER VERMA

I, Dr. Inder Verma, declare as follows:

1. I am the American Cancer Society Professor of Molecular Biology at The Salk Institute, Laboratory of Genetics, La Jolla, California. A copy of my *curriculum vitae* and a list of my publications are attached hereto as **Exhibit A**.

2. I have been retained by the patent owners' and licensee's counsel for the purpose of providing expert technical testimony in this reexamination. I am being compensated at $600.00 per hour (or a maximum of $5,000 for a day). I was previous retained by the licensee's litigation counsel for purposes of the litigation in the District of Massachusetts, but I did not offer testimony in that litigation. I am not otherwise affiliated with the Patent Owners or the licensee.

I. Scope of Opinion

3. I have been provided with, and asked to review, U.S. Patent No. 6,410,516, the Office Action dated August 2, 2006, and the various references cited within that Office Action. I have been asked to provide an analysis of the scientific evidence relied on by the Examiner to reject certain claims of the '516 patent as expressly or inherently anticipated by these references. In particular, I have been asked to provide an analysis as to whether one of skill in the art would have understood these references to describe or disclose the elements of the '516 claims being rejected on the basis of these references. Where the rejection of certain claims has been made on grounds of inherency, I have also been asked to analyze whether there is any basis in fact and/or technical reasoning to support a determination that elements present in these claims would necessarily result from the teachings of the cited art. For the purpose of this declaration I have understood, one skilled in this art in 1989 or even 1991 would have at least a doctoral degree, e.g. a Ph.D. degree, in molecular biology or a related discipline, have at least 3 years of post-doctoral training, have knowledge in cell biology, biochemistry and genetics, and be well trained in laboratory methodologies.

4. The opinions set forth in this declaration are based on my professional knowledge and expertise, as indicated in my curriculum vitae, my review of the '516 patent, application U.S. Serial No. 07/341,436, filed April 21, 1989, and applications incorporated by reference therein,

Orders Granting Requests for Reexamination 90/007,503 and Reexamination 90/007,828, and the Office Action (Merged Request for Reexamination) dated August 2, 2006, including the documents cited in the Office Action, as well as additional documents cited in this declaration. For the purposes of this declaration I have been advised and understand that the claims which will be pending are claims 1-6, 8-27, 29-38, 40-80, 82, 84, and 87-202.

II. Interpretation of the Claims

5. My interpretation of the claims is based on my understanding to how one of skill in the art would have understood the terms appearing in the claims in the context of the claims as a whole, in view of the description of the invention set forth in the patent. To assist in my explanation of the claims, I will refer to a series of 17 explanatory schematics, which are attached to this declaration as **Exhibit 1**.

6. The patent relates to work of the inventors inclduing by Dr. David Baltimore, which grew out of studies on how the immune system is regulated. The immune system consists of a variety of specialized cells, and protects the body by attacking and eliminating harmful foreign organisms and substances. The inventors' work demonstrated that a particular factor in the cell, NF-κB, plays a central role in regulating how immune cells respond to inflammatory stimuli.

7. The inflammatory process involves a series of events that comprise part of the body's protective response to injury and infection. As part of this protective response, various cells respond to inflammatory stimuli by turning on expression of genes having important functions in the immune and inflammatory responses to such stimuli. '516 patent, col. 2, ll. 54-59, col. 12, ll. 57-66, col. 13, ll. 13-29. NF-κB is essential for the proper activation of immune

cells and the inflammatory process and plays a central role in regulating this process. '516 patent, col. 17, ll. 30-37. Specifically, NF-κB functions as a transcription factor, which is activated in response to inflammatory stimuli and participates in regulating expression of particular genes, such as genes encoding antibody chains and cytokines. ('516 patent, col. 2, lines 20-45, col. 12, line 57- col. 14, line 54).

8. In the absence of inducing stimuli, NF-κB is generally present in an inactive form in the part of the cell called the cytoplasm. In its inactive form, NF-κB is tightly bound to an inhibitor protein called IκB, which prevents NF-κB from traveling to the nucleus. Upon activation in response to signals triggered by the interaction of certain external influences with receptors on the surface on the cell, NF-κB is released from its complex with IκB. The released NF-kB then moves to the nucleus of the cell, where it participates in regulating expression of particular genes by binding to specific DNA sequences or "recognition sites" on those genes, leading to transcription of such genes. (Schematic 1 of **Exhibit 1** .) See also, for example, '516 patent, column 2, lines 46-63; column 10, lines 31-55; column 14, lines 28-30; column 16, lines 22-63.

9. While NF-kB activity is essential to allow the body to respond to the effects of harmful external agents, in some cases the activity can become excessive causing other undesirable effects. The entire NF-kB activity pathway from induction to gene expression can be separated into the six (6) general segments shown is Schematic 2 of **Exhibit 1**. The kinetics NF-kB activity induced by the presence of an external influence is represented in blue in the graph in the upper right hand corner of Schematic 2.

10. I understand the claims of the '516 patent to be directed to various methods for intervening in the processes involved in NF-kB activity so as to effect subsequent NF-kB-regulated effects. In situations where there is activation of NF-kB in response to external stimuli, the claimed methods are useful for reducing the potentially harmful effects of these external stimuli. For example, certain pathological states are characterized by the presence of external influences which act on the cell (such as LPS binding to the LPS receptor), which can result in excessive or inappropriate induction of NF-kB activity.

11. The claims are directed to methods for achieving the goal of modifying the naturally occurring response to such external inducing stimuli and reducing the harmful effect of such external influences by "reducing NF-kB activity." For example, see col. 3, lines 59-67, where the patent explains that, "As a result of this finding, it is now possible to alter or modify the activity of NF-kB as an intracellular messenger and, as a result, to alter or modify the effect of a variety of external influences, referred to as inducing substances, whose messages are transduced within cells through NF-kB activity. Alteration or modification, whether to enhance or reduce NF-kB activity or to change its binding activity (e.g., affinity, specificity), is referred to herein as regulation of NF-kB activity." This goal is achieved by reducing the ability of NF-kB to act as a messenger inside the cell so as to effect specific results recited in the claims, such as reducing NF-kB mediated gene expression. When read in light of the teachings of the patent disclosure, one of skill in the art would understand and interpret the claims to require affirmative acts and manipulative steps, calculated to achieve specific results.

12. The '516 patent teaches that one can reduce induced NF-κB activity by interfering anywhere along any of the six (6) segments of the pathway through which NF-κB is activated as

represented by the arrows in Schematic 3 of **Exhibit 1**. One of skill in the art would understand that the '516 patent claims are directed to achieving specified results by interfering along these segments of the NF-kB pathway so as to reduce NF-kB activity.

13. In normal cells, in the absence of inducing stimuli, there is no induced NF-kB activity and therefore no consequent NF-kB mediated intracellular signaling. Such activation and signaling only take place in response to an external influence that acts on the cell. Since the claims recite methods for reducing NF-kB activity, one of skill in the art would understand that the claimed methods do not cover prophylaxis, i.e. in systems not exposed to an induced, but instead are directed to methods such as treating pathological states in which NF-kB activity which has been induced is reduced.

14. I understand some claims, for example, claims 1, 2 and 5, to be directed to methods of achieving specified results by interfering along any one of the six (6) segments shown in Schematic 3. Other claims, for example, claims 8, 9, 10, 14, 95 and 145 require that the external influence "induce NF-kB-mediated intracellular signaling." Thus, these claims require the continuous occurrence of at least the first step, i.e. the induction step 1 which results in intracellular signaling, but encompass interference along any of the remaining five (5) segments inside the cell (Schematic 4 of **Exhibit 1**).

15. Other claims are more specific and require interference with the NF-kB pathway along only one of the five (5) segments which occur within the cell. For example, claims 20, 22, 24, 31, 33, 35, 42, 44 and 46 require interference along segment 2 (Schematic 5 of **Exhibit 1**); claims 23, 34 and 45 require interference along segment 3 (Schematic 6 of **Exhibit 1**); claims 21,

32, 43 and 89 require interference along segment 5 (Schematic 7 of **Exhibit 1**); and claims 25, 47, 80 and 144 require interference along segment 6 (Schematic 8 of **Exhibit 1**).

III. The August 2, 2006 Office Action

16. I note that on pages 3-4, the Examiner stated that none of the claims of the patent are entitled to earlier than a November 13, 1991 filing date. I respectfully disagree. I find the invention described by the claims is also described in U.S. Serial No. 07/341,436, filed April 21, 1989, a copy of which I am advised is attached hereto as **Exhibit 2**. For purposes of the August 2, 2006 Office Action, I have been informed that the issue of the filing date is relevant only for certain claims listed in the Claim Support Chart attached hereto as **Exhibit 3**. I have reviewed the portions of U.S. Serial No. 07/341,436 indicated in the Claim Support Chart, and understand and agree that each of the portions of the application being cited in the chart fully describes the invention recited in the corresponding claim listed in the chart in such a way that a person of skill in the art could readily practice the claimed invention without undue experimentation, i.e. only routine experimentation would be required. I note that on page 1, lines 4-8, U.S. Serial No. 07/341,436 refers to and incorporates by reference three other applications. i.e. 06/817,441, 07/155,207, and 07/280,173. Copies of what I am advised are those applications are attached hereto as **Exhibits 4, 5, and 6, respectively**. I have also reviewed these applications for the purpose of this analysis.

17. Contrary to the Examiner's assertion on the bottom of page 3 of the Office Action, amino acid or nucleic acid sequences corresponding to NF-kB are not recited in the pending claims or necessary for one skilled in the art to practice any of the claims of the '516 Patent as of April 21, 1989. Furthermore, contrary to the Examiner's assertion, I find the phrase "reducing NF-kB activity" to be described in the passage, "Alteration or modification, whether to enhance

or reduce NF-kB activity . . . is referred to herein as regulation of NF-kB activity" on page 5, lines 13-16 of U.S. Serial No. 07/341,436.

18. I find the phrase "mammalian cells" to be described in the passage, "NF-kB [is] present in a wide variety of mammalian cells" on page 77, lines 8-9 of U.S. Serial No. 07/155,207. Similarly, I find the phrase "eukaryotic cells" to be described in the passage "In the present work with eukaryotic cells" on page 4, line 21 of U.S. Serial No. 07/280,173. I note the Examiner also asserted that "'reducing NF-kB activity' in a cell (e.g. especially mammalian/eukaryotic) and/or an enabling means thereof (e.g. administering a NF-kB inhibitor) to effect various functions (e.g. inhibit expression generally, reduce cytokine expression etc.)" is not disclosed in the subject patent. I respectfully disagree. In this regard, I note that, for example, page 23, line 22 to page 30, line 25 of U.S. Serial No. 07/341,436 describes how one skilled in the art can reduce NF-kB activity in accordance with the invention.

19. I find the phrase "reducing binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB" to be described by the passage, "NF-kB-mediated gene expression can also be selectively regulated by altering the binding domain of NF-kB in such a manner that binding specificity and/or affinity are modified" on page 5, line 31 to page 6, line 8; and by the passage, "According to the methods described herein, the expression of genes under the control of one of these elements is subject to modulation by alteration of the concentration or availability of NF-KB. This can also be carried out, according to the present method, for any gene which contains an NF-kB binding site" on page 24, lines 9-29 of U.S. Serial No. 07/341,436, as well as its title.

20. I find the phrase "inhibiting modification of an IkB protein, which modification otherwise reduces IkB binding to NF-kB" to be described by the passage, "Inactive NF-kB is complexed with a labile inhibitor protein, I-kB" on page 18, lines 28-29; by the passage, "The implication is that activation of NF-kB involves a modification of I-kB and not NF-kB" on page 19, lines 20-21; and by the passage "As a result of this finding, it is now possible to alter or modify the activity of NF-KB as an intracellular messenger and, as a result, to alter or modify the effect of a variety of external influences, referred to as inducing substances, whose messages are transduced within cells through NF-KB activity.... In particular, the present invention relates to a method of regulating (enhancing or diminishing) the activity of NF-KB in cells in which it is present and capable of acting as an intracellular messenger, as well as to substances or composition useful in such a method" on page 5, lines 7-13 and 23-25 of U.S. Serial No. 07/341,436.

21. I find the phrase "inhibiting degradation of an IkB protein" to be described in the passage, "Various inducer then cause an alteration in I-kB allowing NF-kB to be released from the complex" on page 20, lines 1-3; and by the passage on page 5, lines 7-13 and 23-25 of U.S. Serial No. 07/341,436. Similarly, I find the phrase "inhibiting dissociation of NF-kB:IkB complexes" to be described by the passage, "... dissociating agents such as formamide and deoxycholate to unmask very high levels of NF-kB activity" on page 19, lines 1-3; and by the passage, "The complex formation of NF-kB with I-kB appears to be rapidly and efficiently reversible ...." on page 20, lines 5-7 of U.S. Serial No. 07/341,436.

IV. Cyclosporin A (CsA)

22. CsA is used as an immunosuppressive drug and is believed to act primarily on T-lymphocytes. The accumulated scientific evidence indicates that CsA exerts its clinical effect through its ability to inhibit an intracellular enzyme called calcineurin. (Loh et al. 1996; Hogan et al. 2003; Ho et al. 1996, **Exhibits 7, 8,** and **9**, respectively). Calcineurin regulates the activity of the NFAT (nuclear factor of activated T cells) transcription factor. Increases in cytoplasmic calcium activate calcinuerin, which by dephosphorylating NFAT, causes NFAT to move to the nucleus. There, NFAT participates in regulating expression of a number of genes, including certain cytokines such as IL-2. By inhibiting calcineurin, CsA can inhibit calineurin-mediated activation of NFAT family members and modulate expression of NFAT regulated genes. (Loh et al. 1996; Hogan et al. 2003; Ho et al. 1996).

A. Express Anticipation Rejection based on Emmel 1989, Schmidt 1990 or Brini 1990

23. I have read claims 1-6, 8, 9, 11, 20-21, 25-27, 29, 35-38, 40, 42-43, 47-51, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89, 93-97, 106-107, 109-110, 114-117, 192-193 and 197-201 that the Examiner has rejected in view of Schmidt 1990, Emmel 1989 or Brini 1990, improved copies of which are attached as **Exhibits 10, 11** and **12**, respectively. All these claims require "reducing NF-kB activity." The Examiner contends that each of these references teach administration of CsA (CsA) to reduce NF-kB activity in cells. The Examiner further contends they teach administration of CsA to reduce NF-kB activity so as to inhibit transcription of NF-kB regulated genes. I respectfully disagree. One of skill in the art would not understand any of these references to demonstrate that use of CsA resulted in reduced NF-kB activity. Moreover, as I discuss in more detail below, I disagree that any of these references describes or teaches any

in view of the '516 patent and other studies, indicates that CsA sensitive binding activity does not correspond to NF-kB activity, but instead corresponds to activity of another transcription factor, NFAT (alternatively "NF-AT"). See, Schematics 11-13 of **Exhibit 1** for reference.

27. In this regard, the Examiner erroneously states that Schmidt used the "same" EMSA assay as the '516 patent. Among other things, Schmidt did not use the same oligonucleotide probe. The particular oligonucleotide probe used as the binding site determines the specificity of the EMSA for measuring NF-kB binding activity under a particular experimental condition. The oligonucleotide probe Schmidt used corresponds to a portion of the HIV enhancer.

28. Notably, the NFAT sequence shares considerable homology with NF-kB in its DNA binding region (Giffin 2003, at 801). A number of studies have demonstrated that NFAT recognizes and binds to a subgroup of the sequences that also serve as NF-kB binding sites. For this reason, without further appropriate controls to confirm the identify of the binding complexes detected, one could not use an EMSA based on a sequence capable of binding both NFAT and NF-kB to discriminate between NF-kB activity and NFAT binding activity. In particular, later studies have demonstrated that one such sequence is the HIV enhancer sequence that was used as an oligonucleotide probe in the Schmidt study. (see, for example, Giffin 2003, Kinoshita et al. 1997, **Exhibits 13** and **14**). In PHA- and PMA-induced Jurkat cells, this EMSA detected at least four complexes with different mobilities, indicating that nuclear extracts from these cells contained multiple factors binding to that HIV enhancer sequence. (Schmidt fig. 1).

29. Recognizing the apparent lack of specificity indicated by their EMSA data indicated, the Schmidt authors carefully qualified their conclusions, characterizing the complexes

bound to the HIV enhancer only as being "NF-kB-like," and not as being NF-kB (Schmidt at 4039). The Examiner's conclusion that the CsA-sensitive complex Schmidt observed corresponds to NF-kB therefore overstates what the authors themselves concluded about their own data.

30. The '516 patent similarly used EMSA to analyze binding activity in Jurkat cells induced with PMA and/or PHA. ('516 patent, fig 24A). However, instead of the HIV enhancer, that EMSA used a portion of the kappa immunoglobulin enhancer (kappa 3)('516 patent col. 72, line 54-58; fig. 13A, col. 50 line 8-22, col. 50, line 60 – col. 51, line 5). EMSA analysis demonstrated that PMA alone induced significant NF-kB binding activity in Jurkat cells. ('516 patent, fig 24A). The kappa 3-based EMSA detected a single specific band, reflecting the greater specificity of that EMSA for NF-kB in these cells. In contrast, as evident from the experiment reported in Fig. 24, PHA alone did not induce NF-kB binding activity in Jurkat cells. ('516 patent, fig. 24A).

31. In view of the '516 patent, one of skill in the art would therefore not interpret the PHA-induced binding to the HIV enhancer Schmidt observed in Jurkat cells as NF-kB binding activity. The observation by Schmidt that PHA-induced binding to an HIV enhancer probe was CsA sensitive therefore does not demonstrate that (as the Examiner assumes) CsA reduced NF-kB activity. Indeed, when Schmidt assessed induction of NFAT binding activity in Jurkat cells using an EMSA specific for NFAT, (Schmidt, fig. 2), there was "good activation of NFAT-1 with PHA alone, but not with PMA alone." Such data explain the positive EMSA binding activity induced by PHA treatment that Schmidt observed with the HIV enhancer likely corresponds to NFAT.

32. Further, the '516 patent indicates that in contrast to PHA, PMA induced substantial NF-kB binding activity in Jurkat cells. ('516 patent, Fig. 24B). However, in Schmidt, CsA did not prevent PMA-induced binding activity to the HIV enhancer in Jurkat cells. Therefore, to the extent PMA-induced binding activity to the HIV enhancer that Schmidt observed could be interpreted to correspond to NF-kB, Schmidt's data indicate that CsA does not prevent PMA from inducing NF-kB binding activity in Jurkat cells. These data provide further evidence that in PHA-induced Jurkat cells, the only binding activity affected by CsA corresponds to the activity of NFAT.

33. The Examiner also relies on CAT reporter data in Schmidt as demonstrating that CsA affects NF-kB activity. I disagree. While the '516 patent describes use of transfected cells as one of several assays for measuring NF-kB activity, the claims are not directed to methods for carrying out these assays. In particular, claims 1-6, 8, 9, 11, 20-21, 25-27, 29, 35-38, 40, 42-43, 47-51, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89, 93-97, 106-107, 109-110, 114-117, 192-193 and 197-201 are all directed to methods in human, mammalian or eukaryotic cells. As one of skill in the art, I interpret such cells as those that one would normally find in a human, mammal or eukaryote. The experiments using CAT reporter assays all employed artificial transfected cells bearing foreign bacterial DNA. Such artificial, transfected cells would not normally be found in any human, mammal or eukaryote. Therefore, none of the CAT reporter experiments in Schmidt would involve any of the claimed methods of the '516 patent.

34. Moreover, to the extent they provide information about the effect of CsA, the CAT reporter assay data further suggest that CsA does not affect NF-kB activity in Jurkat cells. Schmidt used a CAT reporter construct engineered to place a bacterial CAT gene under the control of sequences found in the HIV LTR. (Schmidt fig. 4). Significantly, CsA failed to

prevent PMA from inducing CAT gene expression. (Schmidt fig. 4, page 4039, col. 1). As the '516 patent indicates PMA would induce NF-kB binding activity in Jurkat cells, these data provide additional evidence that CsA does not affect NF-kB activity. In particular, Schmidt's data that CsA did not affect PMA-induced expression of the CAT reporter indicate CsA would not have reduced NF-kB mediated gene expression in Jurkat cells.

35. The fact that Schmidt observed that CsA partly prevented PHA-induced CAT expression does not indicate that CsA did so by reducing NF-kB binding activity. As noted above, the data in Schmidt show that in Jurkat cells, the HIV enhancer sequence binds other factors. Additionally, '516 patent Fig. 24 indicates that PHA alone fails to induce measurable amounts of NF-kB binding activity in Jurkat cells. From these data, one of skill in the art would conclude that the effect of CsA Schmidt observed on expression of the bacterial CAT gene did not involve NF-kB, but was likely mediated by some other transcription factor such as NFAT.

36. The Examiner further contends that "use of the HIV LTR gene by Schmidt" renders obvious claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192, and 197-201 by describing a method "to regulate NF-kB activity as in Schmidt in order to affect associated viral (e.g., HIV) gene expression." I respectfully disagree. For the reasons discussed above, one of skill in the art would understand that CsA mediates its effects through NFAT, not through NF-kB, and would not understand Schmidt as suggesting any method of using CsA to regulate NF-kB activity in cell in order to reduce expression of any gene, e.g. a viral gene.

(ii) Express anticipation rejection based on Emmel

37. Similarly, Emmel reports experiments conducted in Jurkat cells, a human T cell lymphoma line. Emmel examined the extent to which simultaneous treatment with CsA

appeared to prevent certain responses in these cells induced by a combination of PHA and PMA. The Examiner contends that the use of CsA as described in Emmel would anticipate '516 patent claims 1-6, 8, 9, 11, 20-21, 25-27, 29, 35-38, 40, 42-43, 47-51, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89, 93-97, 106-107, 109-110, 114-117, 192-193 and 197-201, if these claims were entitled only to a November 13, 1991 filing date. I respectfully disagree.

38. As noted above, each of the above claims are directed to methods carried out on cells in which NF-kB activity has been first induced, since only then can it be reduced. The Examiner states that Emmel "describes effects of CsA on Jurkat cells that *were* induced with [a combination of] PHA and PMA." (emphasis added). This does not accurately describe the experiments Emmel conducted. In the experiments (Figs. 2D, 3) the Examiner relies on, CsA was not administered to Jurkat cells that had been induced with PHA/PMA. Instead, Jurkat cells were simultaneously stimulated with PHA/PMA and CsA. In these experiments, CsA could not have reduced signaling that was occurring. None of these experiments would therefore have carried out the method recited in any of the claims.

39. The Examiner relies on CAT data "as shown in Fig. 2D" to contend that "CsA significantly reduced NF-kB activity" thereby reducing NF-kB-mediated gene expression (i.e, CAT expression) (Office Action at 16). I respectfully disagree. As in Schmidt, this CAT reporter assay used a construct bearing a portion of the HIV LTR linked to a heterologous promoter to drive expression of the bacterial CAT gene. The Examiner's conclusion that this experiment shows that CsA reduced NF-kB activity is erroneous. Emmel acknowledges the lack of any conclusive evidence that NF-kB is involved in any of the effects that CsA has through the HIV LTR: " a binding site for NF-kB is present in the long terminal repeat of the human immunoglobulin deficiency virus (HIV-LTR)(17), yet CsA sensitivity of the HIV-LTR is

independent of this site (18)." (Emmel at 1619). As discussed above regarding Schmidt, most likely, CsA affected CAT expression by reducing NFAT binding, and not through any effect on NF-kB.

40. Emmel describes additional CAT assay data indicating that NF-kB does not mediate effects of CsA on gene expression. Using a CAT reporter construct based on the IL-2 enhancer, Emmel "tested the effect of CsA on a series of internal deletion mutations of the IL-2 enhancer" (Emmel at 1618). Emmel observed that CsA "virtually eliminated" PHA/PMA-stimulated CAT expression driven by the intact IL-2 enhancer. CsA effectively prevented PHA/PMA-stimulated expression of CAT reporter driven by tandem repeats of an NFAT binding site. (Emmel, Figure 2B). However, it is significant, that deleting the NF-kB binding site did not affect the ability of CsA to prevent PHA/PMA from inducing CAT expression. (Emmel at 1618, Figure 1). These data provide strong evidence that NF-kB does not play any role in mediating the effect of CsA on IL-2 gene expression. Indeed, these data in Emmel confirm our current understanding that in Jurkat cells, CsA acts through NFAT, not through NF-kB.

41. The Examiner also relies on EMSA data "as shown in Fig. 3" purportedly showing that "CsA was found to reduce binding activity." I disagree with the Examiner's assertion that these data demonstrate that CsA reduced NF-kB activity in Jurkat cells as recited in the claims. Emmel observed that CsA had only a small (of about 10-20%) apparent effect on preventing PMA/PHA-induced binding activity, only at the highest CsA concentrations, and did not demonstrate any dose response. As discussed above, as used in these studies, the oligonucleotide probe would not have discriminated between NF-kB and NFAT binding activities. Accordingly, one can see multiple bands appearing in extracts from PHA/PMA treated cells under the conditions Emmel used. (Emmel Fig. 3). (For example, compare with '516 patent, Fig. 24B

indicating only a single inducible band in PHA/PMA-treated Jurkat cells). Appropriate controls would have been critical for the correct identification of the different complexes bound to the probe, and to the overall interpretation of the EMSA data. Such a control could have been carried out, for example, by using a probe carrying a mutated binding site, or through methylation interference analysis. ('516 patent, col. 20, line 20-25; col. 22, line 32-39).

42. Significantly, Emmel did not include such controls. These data are insufficient to indicate if any of the different complexes detected in the EMSA correspond to NF-kB, cannot demonstrate that the CsA sensitive binding activity corresponded to NF-kB. Therefore one cannot conclude from Emmel that CsA affected NF-kB binding activity. Today, we know that the likely effect of CsA on NFAT provides the simplest and most likely explanation for these inconclusive data obtained by Emmel.

43. It is also significant that the authors of Emmel questioned the relevance of their EMSA data to NF-kB. In particular, Emmel notes that these observations "may not be related to the mechanism of action of the drug, since the CsA concentration dependence of inhibition of the appearance of NF-kB binding does not correlate well with the CsA concentration dependence of T cell activation." (Emmel at 1619).

(iii) Express anticipation rejection based on Brini

44. The Examiner contends that Brini teaches administration of CsA to cultured T lymphocytes, which substantially reduced NF-kB activity in those cells thus inhibiting expression of genes whose transcription is regulated by NF-kB activity. The Examiner contends that the use of CsA as described by Brini would practice claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29,

36-38, 40, 53-54, 58-62, 65-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97, if these claims were entitled only to a November 13, 1991 filing date. I respectfully disagree.

45. Brini reports results of certain experiments conducted with isolated populations of human peripheral blood cells that had been enriched for T lymphocytes. (Brini at 132). Brini examined the effect of CsA on the ability of PHA (phytohemaglutinin) to induce certain responses in these cells. Notably, in all experiments, "CsA was always added to the cells 30 min before stimulation" with PHA. (Brini at 132). In this regard, the Examiner erroneously states that Brini disclosed use of CsA "in human PBM (peripheral blood T-lymphocytes) that *had* been induced with PHA." (Office Action at 17).

46. As an initial matter, I understand all the above claims to be directed to methods carried out on cells in which NF-kB activity has first been induced by some external stimulus. However, none of Brini's experiments examined the effect of CsA in cells first induced with PHA. Therefore, the method of using CsA that Brini describes does not conform to any of the claims.

47. The Examiner contends that Brini "discloses that administration of CsA reduces NF-kB in cells (e.g., T cells) that inherently reduces NF-kB regulated gene expression." (Office Action at 17). The examiner specifically relies on EMSA binding assays (Figs. 3 and 4) and data that CsA caused a small apparent decrease (approximately 40-50%) in the ability of PHA to induce IL-2 receptor-alpha expression. I respectfully disagree that the data which Brini provides supports the Examiner's conclusion.

48. The EMSA assays Brini describes used an oligonucleotide probe corresponding to a sequence in the IL-2 receptor promoter, or a sequence corresponding to a portion of the HIV

LTR. In both EMSA assays, Brini observed "several discrete-retarded DNA-proteins" (both in PHA-treated cells as well as untreated cells) that bound to the probe. (Brini at 135). Brini reports that CsA had a small apparent effect on increase in binding of some of the bound complexes. (Brini at 135).

49. Neither of the EMSA assays Brini conducted demonstrates whether the CsA sensitive binding activity corresponds to NF-kB. As discussed above, and evident from the data of Schmidt and Emmel, CsA can potently affect NFAT binding activity. Moreover, NFAT recognizes and binds to certain sequences that also serve as NF-kB binding sites. For these reasons, it is critical to include appropriate controls in EMSA assays conducted to evaluate the potential effect of CsA on NF-kB binding activity to confirm the identity of any CsA-sensitive binding complexes and discriminate between NF-kB activity and NFAT binding activity. The lack of any such controls in the experiments Brini conducted makes it impossible to conclude that any of the complexes affected by CsA (Figs 3 and 4) correspond to NF-kB. Without such data, it is also impossible to conclude from the EMSA data in Brini that PHA induced NF-kB activity. In fact, the title of the Brini's reference refers only to "NF-kB-like factor(s)" and not to "NF-kB."

50. The fact that Brini observed a substantial level of binding activity in untreated cells further argues that it is unlikely that the complexes Brini observed correspond to NF-kB, because one would not observe NF-kB activity in uninduced cells. (For example, see '516 patent, Fig. 24). Moreover, CsA alone appears to increase levels of the complexes that Examiner interprets correspond to NF-kB. (Fig. 3, lane 2). In particular, the assumption that these complexes correspond to NF-kB is inconsistent with the premise that in the experiments Brini conducted, NF-kB activity regulated IL-2 receptor expression, because there is no correlation between the

presence of these complexes in the EMSA assays (Figs. 3 and 4) and IL-2 receptor mRNA expression (Fig. 2).

51. The Examiner further relies on data indicating that CsA (in cells pretreated with CsA) slightly decreased (by approximately 40-50%) the ability of PHA to induce IL-2 receptor-alpha expression. The Examiner contends these data demonstrate that CsA reduced NF-kB regulated gene expression. In support, the Examiner contends that "expression levels of IL-2 Receptor-alpha is taught by the '516 patent to be regulated by PHA-induced NF-kB activity in T cells." (Office Action at 18). I respectfully disagree. First, the Examiner's interpretation of the '516 patent is erroneous. The statement the Examiner cites notes that "NF-kB is induced in T cells...by PMA/PHA treatment and thereby activates the IL-2 receptor alpha gene..." (Office Action at 18). These data provide no evidence as to whether PHA treatment alone would induce NF-kB activity so as to induce IL-2 receptor expression.

52. As the '516 patent, as well as Emmel and Schmidt illustrate, T cells can exhibit substantial differences in their response to PHA and PMA. As noted above, PHA alone did not induce measurable amounts of NF-kB binding activity in the Jurkat T-cell line. ('516 patent, fig. 24A). Without data, the Examiner has no basis to assume that treatment of T-cells with PHA alone (as compared to treatment with both PHA and PMA) would similarly induce NF-kB activity so as to induce expression of any gene. In fact, Brini reports that PHA appeared to induce binding activity of at least one other transcription factor, AP1, having binding sites in the IL-2 receptor promoter. (Fig. 5). Lastly, as noted above, even assuming the complexes Brini detected by EMSA (Figs. 3 and 4) correspond to NF-kB, there is no correlation between the presence of these complexes in the EMSA assays (Figs. 3 and 4), and IL-2 receptor mRNA expression (Fig. 2). These data therefore fail to provide any basis for concluding that under the

experimental conditions which Brini used, IL-2 receptor expression was regulated in any way by NF-kB.

B. Inherent Anticipation Rejection Based on Reed 1986, Kronke 1984 or Siebenlist 1986

(i) Reed 1986

53. The Examiner contends that Reed teaches use of CsA in human peripheral blood mononuclear (PBM) cell cultures, and that Brini explains that such use inherently reduces NF-kB activity and thus expression of genes whose transcription is regulated by NF-kB activity. The Examiner contends that Reed inherently anticipates claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31-32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97. I respectfully disagree.

54. Reed reports results of certain experiments conducted with primary cultures of human peripheral blood mononuclear (PBM) cells. Among other things, Reed examined the effect of CsA on the ability of PHA (phytohemaglutinin) to induce certain responses in these cells, including cell surface expression of the IL-2 receptor, and expression of IL-2 receptor-α mRNA. The authors note that in all experiments, CsA was always added to the cells 20 to 30 minutes before other reagents (which included PHA). Reed, at 150. In this regard, the Examiner erroneously states that Reed "taught the prior art use of CsA in human PBM cultures that *had been induced* with phytohaemaglutinin (PHA). (Office Action at 25).

55. As noted above, I understand the '516 patent claims are directed to methods carried out on cells in which NF-kB activity has first been induced by some external inducing stimulus. In contrast, none of Reed's experiments described use of CsA in cells that were first induced with PHA. Therefore, regardless of any effects that CsA might have had on NF-kB (for which

there is no evidence), none of the methods of using CsA that Reed discloses involve reducing either induced NF-kB activity or induced NF-kB-mediated intracellular signaling. Therefore, none of the uses described by Reed described the method recited in any of the claims.

56. Further, I disagree with the Examiner's conclusion that Brini demonstrates that any method of using CsA described by Reed would inherently reduce NF-kB activity and thus expression of genes whose transcription is regulated by NF-kB activity. As discussed above, none of the data in Brini demonstrate that CsA either reduced NF-kB activity, or provide any basis for concluding that under the experimental conditions Brini used, IL-2 receptor expression was regulated in any way by NF-kB. In this regard, I disagree with the Examiner that Brini demonstrates any inhibition "done by reducing binding of NF-kB to NF-kB recognition sites." Moreover, the Examiner's statement that Brini also demonstrates that use of CsA also resulted in "inhibiting the passage of NF-kB into the nucleus of cells, inhibiting modification of an IkB protein, and inhibiting degradation of an IkB protein," is entirely unsubstantiated by any experimental evidence. (Office Action at 24). Brini has no data regarding IkB, let alone any data regarding degradation or modification of IkB. Brini also does not provide data demonstrating that CsA inhibited translocation of NF-kB into the nucleus.

57. Morever, Brini does not replicate any of the experiments Reed conducted. Therefore, Brini cannot explain or confirm what occurred in Reed. For example, the studies Brini describes were conducted using a substantially different cell population than that which Reed used. Therefore Brini did not use "the same immune cells" as Reed. (Office Action at 26).

58. Reed used cultures of PBM cells isolated by Ficoll-Hypaque density gradient purification. (Reed at 150). "PBM cells" does not refer to a single, defined cell type. Such cell

cultures comprise a highly heterogeneous cell population comprised of numerous immune cell types, including T and B-lymphocytes, NK (natural killer) cells, monocytes and macrophages. (See for example, David et al. Blood (1998), **Exhibit 15** hereto). There is substantial variability in PBM cells, depending on the protocol and conditions of isolation. Reflecting this fact, studies have reported conflicting data in PBM cells regarding the particular endpoint Reed measured, IL-2 receptor-α expression. (David et al. 1998 at 165, 170-171). Among other things, IL-2 receptor-α expression is highly sensitive to the conditions of blood collection and PBM preparation, and varies substantially among the various subtypes of cells found in PBM cultures. (*Id.*). Such data demonstrate the importance of closely replicating experimental conditions if one were to attempt to assess what might have occurred in some earlier study using "PBM" cells.

59. In contrast to Reed, Brini used cultures comprised primarily (>95%) of CD3 positive T-lymphocytes, which were isolated through additional purification steps, including nylon wool and Percoll density-gradient purification. (Brini at 132). Such cell cultures are substantially different from the mixed cell population used in Reed, both in the conditions of preparation and in their composition. In this regard, I note that the Examiner erroneously stated on page 26 of the Office Action that Brini conducted experiments in the "same immune cells." Therefore, even assuming that under the experimental conditions Brini used CsA was capable of reducing NF-kB activity, one of skill in the art would not reasonably conclude that CsA necessarily had the same effect on the substantially different cell population Reed used.

60. In support of his arguments regarding Reed, the Examiner also contends that "expression levels of IL-2 Receptor-alpha is taught by the '516 patent to be regulated by PHA-induced NF-kB activity in T cells." (Office Action at 26). As noted before, the Examiner's interpretation of the '516 patent is erroneous as the passage the Examiner cites to notes only that

"NF-kB is induced in T cells...by PMA/PHA treatment and thereby activates the IL-2 receptor alpha gene..." (Office Action at 25). As discussed above, the data in the '516 patent indicate that treatment of T cells with PHA alone does not result in any measurable activation of NF-kB, in contrast to treatment with PMA, or PMA in combination with PHA. Moreover, the '516 patent examples the Examiner refers to (Fig. 24) were conducted with a human T-cell lymphoma cell line, and not under the experimental conditions of Reed using PBM cells. Regardless, we know now that CsA affects the activity of NFAT, not NF-kB.

(ii) Kronke 1984 or Siebenlist 1986

61. The Examiner contends that the use by either Kronke or Sieblenlist of CsA in cell cultures, as explained by Schmidt and Emmel, inherently reduces NF-kB activity and "thus would inhibit expression of genes whose transcription is regulated by NF-kB activity." The Examiner therefore contends that either Kronke or Siebenlist inherently anticipates claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31-32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97. I respectfully disagree.

62. Kronke and Siebenlist report experiments in various cells, including the Jurkat T cell line, examining various effects of CsA on IL-2 gene expression. In particular, the Examiner relies on certain experiments in which cells were also treated with PHA and PMA. (Office Action at 28, Kronke at 5217, Siebenlist at 3044). As discussed above, neither Schmidt nor Emmel describe any use of CsA in cells that reduced NF-kB activity or resulted in any NF-kB mediated effect. Therefore, neither of these studies provides any basis for concluding that such effects would necessarily have occurred during the use of CsA described in either Kronke or Siebenlist.

63. Regarding the Examiner's statements regarding Kronke and Sieblenlist, I note additionally the following. None of experiments in Siebenlist describe use of CsA in cells first induced with PHA and PMA. Therefore, regardless of any effects that CsA might have had on NF-kB (for which there is no evidence), none of the methods of using CsA that Sienbenlist describes would have reduced either induced NF-kB activity or induced NF-kB-mediated intracellular signaling. Kronkė describes only a single experiment in which cells were treated with CsA after exposure to PHA/PMA, and concludes in the title of Table 1 "CsA does not inhibit PHA- and PMA- induced expression of TCGF receptors in Jurkat cells." Moreover, as is evident from Fig. 4, CsA treatment appeared to have a significant effect on IL-2 expression only when CsA was added either before or with PHA/PMA treatment, but not when added after cells had been induced with PHA/PMA. Kronke at 5217. Indeed, when added four hours after induction, CsA did not alter TCGF (IL2) mRNA levels. Id. at 5216. These data suggest that while treatment with CsA appeared able to prevent induction of IL-2 expression, it had no substantial effect on induced IL2 expression. Regardless, we know now that CsA affects NFAT activity, not NF-kB.

(iii) Inherent anticipation rejection based on the PDR 1985, Griffith 1981 and Griffith 1984

64. The Examiner contends that the PDR 1985 (pages 1811-13), Griffith 1981 or Griffith 1984 teach administration of CsA to cells, which based on Holschermann, would inherently reduce NF-kB activity and therefore expression of NF-kB regulated genes. The Examiner contends that each of these references inherently anticipate claims 1-2, 5, 6, 8, 9, 20-27, 29, 31-40, 64-73, 75-80, 82, 84 and 88-97. I respectfully disagree.

65. Griffith 1981 and Griffith 1984 both describe various clinical studies conducted to evaluate use of CsA in transplant patients. The 1985 PDR describes potential use of CsA in transplant patients. None of these references describes method of using CsA that would carry out the method recited in any of the claims. None of these references mention NF-kB, describes any external influence that would necessarily induce NF-kB activity or NF-kB mediated intracellular signaling or describe any effect of CsA necessarily resulting from or mediated through NF-κB. Moreover, I disagree that Holschermann provides any basis for concluding that such elements would necessarily have occurred in any prior use of CsA as described by Griffith 1981 or 1984, or the 1985 PDR.

66. As noted above, I understand the above claims to be directed to methods carried out on cells in which NF-kB activity has first been induced by some inducing stimulus (external influence). Neither of the Griffith studies, nor the 1985 PDR identifies an external influence that would have necessarily induced NF-kB activity. Moreover, in the patient studies reported in Griffith 1981 and 1984, CsA was administered before transplantation. As the Examiner indicates, the 1985 PDR teaches CsA should be administered first before and then after surgery. There is no evidence that any of these methods of using CsA described in the pre-April 21, 1989 references art would necessarily involve administration of CsA in cells in which NF-kB activity has first been induced.

67. As discussed above, the accumulated scientific evidence indicates that CsA exerts its clinical effect as an immunosuppressant through its effect on transcriptional factor NFAT. The only potential effect of CsA on gene expression which the 1985 PDR mentions is on expression of IL-2, which the data discussed above indicates is mediated not through NF-kB but through NFAT. The Examiner nevertheless contends that Holschermann demonstrates that

CsA, as used in Griffith and/or described in the 1985 PDR would have inherently reduced NF-kB activity. I disagree.

68. First, there is no showing in the PDR that the use of CsA in patients followed exposure to an external stimulus that would necessarily induce NF-kB activity in cells of those patients, such that the induced NF-kB activity could be reduced as required by the claims. Similarly, in the two Griffith references, there is no evidence indicating what, if anything had necessarily first induced NF-kB in the cells of patients in the studies reported.

69. Even if Holschermann had demonstrated that in transplant patients, CsA had some effect on NF-kB (and I disagree that it does), Holschermann fails to replicate the Griffith studies and therefore it is an error to assume that CsA necessarily had the same effect in the patients in Griffith. In this regard, I note that the Examiner bases his premise that CsA would have had the same effect on the erroneous statement that Holschermann "essentially repeated the tests disclosed in Griffith". (Office Action at 25). This is not correct. Several key differences exist between Griffith's studies and Holschermann that would preclude such an assumption. In contrast to patients in the Griffith studies, patients in the Hoschermann study all received additional drugs, including azathioprene, and aspirin, which were not provided to the Griffith patients. (Holschermann at 4232, Griffith 1984 at 952, Griffith 1981 at 325). Whereas patients in Holschermann's study all received antithymocyte globulin, in Griffith 1984 this drug was provided only for rescue from severe or chronic tissue rejection. Moreover, the timing of CsA administration, which in the Griffith studies was first administered before transplantation, differed significantly from Holschermann, where CsA treatment took place only after transplantation. Holschermann at 4233.

70. Further, none of the data Holschermann reports demonstrate that CsA had any effect that in patients that was mediated by or resulted from any effect of CsA on NF-kB. The Holschermann study used an enzymatic assay to measure tissue factor (TF) activity in peripheral blood mononuclear cells (PBMCs) isolated from ten cardiac transplant patients being treated with CsA. The authors observed that TF activity appeared to be reduced in PMBCs isolated from some, but notably, not from all patients after being administered CsA. Holschermann, Fig 2B. The authors indicate that the apparent inhibition may have, but did not necessarily, result from a direct effect of CsA on monocytes. The authors speculate that CsA had certain effects in these patients that the authors suggest were mediated by reducing NF-kB binding activity in monocytes, in particular, on TF expression. I disagree that these data support such a hypothesis.

71. The only data relating to transcription of the TF gene in patients (presented in Figure 3) fails to demonstrate any link between NF-kB activity and TF expression. In particular, Figure 3 reports TF mRNA levels in cells directly isolated from patients before CsA treatment (lane 2) and after treatment (lane 5). Holschermann purportedly found significant activation of NF-kB in mononuclear cells directly isolated from patients before CsA treatment (Figure 4), but at the same time, did not observe detectable TF mRNA expression in such cells. (Figure 3, lane 2). These data show that *in vivo*, there was an apparent lack of correlation between purported NF-kB activity and transcription of the TF gene in the mononuclear cells of the transplant patients. Therefore, these data fail to support the inference that in transplant patients, TF gene expression is necessarily mediated by NF-kB or caused by induced NF-kB activity.

72. Even assuming the data in Holschermann to be valid, the only data showing some effect of CsA on transcription of the TF gene were obtained through *in vitro* experiments, which involved further manipulation and treatment of monocytes originally isolated from patients.

Neither the Griffith studies, nor the 1985 PDR describe any *in vitro* manipulation of cells. Such experiments in Holschermann therefore do not replicate any method of using CsA described in either Griffith paper or in 1985 PDR, nor do they reflect what went on in the cells of patients in the Griffith studies.

73. Moreover, in my opinion, the Holschermann study provides insufficient data to demonstrate that in patients, CsA treatment reduced NF-κB binding activity in monocytes. The only data relating to the effect of CsA on NF-kB binding is a single binding assay reported in Figure 4. This data suffers from several flaws. The paper provides no indication whatsoever on how many patients these data are based on, or the degree to which the effect was reproducible from patient to patient. Moreover, based on the data Holschermann provides, the apparent decrease in binding in extracts from CsA treated patients cannot be interpreted as showing decreased binding of NF-κB. Several studies (see, for example, Giffin 2003, Kinoshita et al. 1997) have demonstrated that the consensus sequence that Holschermann used in the EMSA to assess binding of NF-kB binds other transcription factors, such as NFAT. In view of these other data, the EMSA binding assay Holschermann used lacked sufficient controls, such as an appropriate antibody control, to confirm that the binding complex reported as being affected by CsA was in fact NF-κB. As the data in Figure 4 is insufficient to demonstrate that CsA reduced NF-kB binding in the patients Holschermann studied, one could not reasonably infer from this study that NF-κB activity was reduced in all patients during prior use of CsA as described in 1985 PDR, Griffith 1981 and Griffith 1984.

74. Further, Holschermann does not provide a sufficient basis from which one could infer that the patients studied were characteristic of transplant patients treated with CsA as described in the cited references published before 1989. Both Griffith 1981 and Griffith 1984

note the effects of CsA *in vivo* can vary from patient to patient in part from the high incidence of toxicity and various side effects, and considerable variability in blood and tissue levels. Holschermann observed "a high inter-individual variation in monocytes TF inducibility." (p. 11). Additionally, Figure 2 (lower panel) indicates that CsA treatment did not reduce TF activity in all patients. These other variables further indicate that even had Holschermann replicated experiments in Griffith or the PDR, one could not conclude that the results Holschermann observed would necessarily be observed in any patient treated with CsA in the manner described in 1985 PDR, Griffith 1981 and Griffith 1984. Most importantly, we know now that CsA affects NFAT activity, not NF-kB activity.

V. Inherent Anticipation Rejection Based on Art Relating to Glucocorticoids (1970 PDR, Nagasawa (1981) or Rovera (1979))

75. I understand that the Examiner has rejected claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31-32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97 as anticipated by Nagasawa 1981, Rovera 1979, or selected portions from the 1970 PDR, and explained by various references published after April 21, 1989. (Office Action at 34-35). I have reviewed each of these references, and the above claims. The Examiner contends that each of the references published before April 21, 1989 teaches administration of glucocorticoids (e.g., dexamethasone) to treat inflammatory and allergic diseases such as asthma and arthritis (presumably the 1970 PDR), and in human cell culture tests (Nagasawa and Rovera). The Examiner contends that cited later references is extrinsic evidence where shows that administration of glucocorticoids as described in the earlier references would have necessarily reduced NF-kB activity and concomitant cytokine production. The Examiner contends that each of these references inherently anticipates the above claims. I respectfully disagree.

(i) Inherent anticipation rejection based on Nagasawa (1981) or Rovera (1979)

76. Each of the above claims requires "reducing NF-kB activity." As described above, I understand all the above claims are directed to methods carried out on cells in which NF-kB activity has first been induced by some external inducing stimulus. Therefore, some claims (e.g. claims 1, 2 and 5) encompass both extracellular and intracellular interference with the NF-kB pathway; other (e.g. claims 8 and 9) encompass only intracellular interference with the NF-kB pathway. Neither Nagasawa nor Rovera describe any such method of using glucocorticoids. Therefore, uses of glucocorticoids described in these references would not involve the method recited in any of the claims (Schematic 16 of **Exhibit 1**).

77. In particular, the Examiner states that "Nagasawa taught induction of human leukemic T-Cells (MOLT-3) by TPA, *followed by* the administration of 10^{-6} (1uM) dexamethasone." (Office Action at 35) (Emphasis added). This statement mischaracterizes Nagasawa. Nagasawa describes only one experiment relating to the use of dexamethasone in MOLT-3 cells and finds no effect. (See page 185, Table 2). In this experiment, dexamethasone was administered to MOLT-3 cells either 1 day before or simultaneously with administration of TPA. It was never administered following induction of TPA. Therefore, Nagasawa does not describe the use of dexamethasone in cells that have been induced with TPA, and therefore does not describe the use of dexamethasone in cells that have NF-kB activity. Accordingly, there is no NF-kB activity to be reduced, and no NF-kB activity reduced, upon administration of dexamethasone.

78. The Examiner also states that "similarly, Rovera taught the induction of human leukemic cells (promyelocytes and mature myeloid cells) with TPA, *also followed by*

administration of 10^{-6} dexamethasone." (Office Action at 35) (Emphasis added). This statement similarly mischaracterizes Rovera. Rovera describes only a single experiment (in which apparently only two data points were collected) relating to administration of dexamethasone in the HL-60 tumor cell line. (Rovera page 869, Table 1). In this experiment, dexamethasone was administered to HL-60 cells either 24 hours before or simultaneously with administration of TPA. It was not administered following induction with TPA. Therefore, Rovera also does not describe the use of dexamethasone in cells that have been induced with TPA or that have NF-kB activity which could be reduced.

79. Neither of these references provides any evidence demonstrating that TPA necessarily induced NF-kB activity in the cells studied. Nagasawa describes induction of certain morphological and biochemical markers of differentiation in MOLT-3 cells. (Nagasawa at 182). After four days of simultaneous exposure to TPA and dexamethasone the authors measured the percentage of E-rosette positive MOLT-3 cells. There is no evidence (including that in Exhibit H-8, incorporated into the August 2, 2006 Office Action) showing that any of these induced markers relate in any way to NF-kB. Further, there is no evidence (including that in Exhibit H-8) that in MOLT-3 cells, TPA necessarily induced expression of any particular gene, let alone any gene regulated by NF-kB.

80. Rovera describes the ability of TPA to induce certain morphological changes in HL-60 cells, as well as adherence of the cells to substrate. There is no evidence (including that in Exhibit H-8) showing that these changes in morphology or adherence relate in any way to NF-kB. Further, there is no evidence (including that in Exhibit H-8) that in HL-60 cells, TPA necessarily induced expression of any particular gene, let alone any gene regulated by NF-kB.

81. Finally, none of the post filing references which purports to be extrinsic evidence of inherency that the Examiner cites (Auphan, Scheinman I and II, Mukaida or Padgett) demonstrates that either Nagaswa or Rovera describes a method of (a) using glucocorticoids in cells in which NF-kB activity has been induced or (b) reducing induced NF-kB activity. To the extent that the Examiner has interpreted these post-filing references as evidence that glucocorticoids (in particular, dexamethasone) "are recognized to necessarily and inherently reduce NF-kB activity and concomitant cytokine production," these references do not support such a statement.

82. Moreover, none of these later references reproduces any experiment in Nagasawa or Rovera. As discussed below, there is considerable uncertainty relating to the potential mechanism of action of glucocorticoids in cells, including whether there are any potential effects on NF-kB, as reflected in a substantial amount of contradictory data reported in the scientific literature. Therefore, careful replication would be required to make any prediction as to what effects glucocorticoids may have had in earlier studies. None of the post-filing references provide such data or make any attempt to do so.

83. The Examiner appears to assume that TPA necessarily has the same effect in all human leukemic cell lines. This assumption is erroneous as evidenced by Nagasawa, which points out that "in addition to being able to elicit varying responses in different experimental systems, these tumor promoters can also have contrasting effect on cells apparently derived from the same lineage of differentiation." (Nagasawa at page 190).

84. In the study by Nagasawa, the authors measured the percentage of E-rosette positive MOLT-3 cells after four days of simultaneous exposure to TPA and dexamethasone.

The only conclusion Nagasawa makes regarding glucocorticoids in MOLT-3 cells is that administration had no effect on induction of MOLT-3 cells by TPA. (Nagasawa at 185). Similarly, the only conclusion that Rovera makes regarding glucocorticoids in HL-60 cells is that dexamethasone did not "block[] the response of HL-60 cells to TPA, even when the cells were pretreated with a concentration of the test compound 1000 times greater that the concentration of TPA used to induce differentiation." (Rovera at 869). Such evidence of lack of effect cannot demonstrate that administration of glucocorticoids either reduced NF-kB activity in the cells or had any effect in the cells that was mediated by or resulted from an effect on NF-kB.

(ii) Inherent anticipation rejection based on the 1970 PDR

85. The section of the 1970 PDR cited in the Office Action provides that dexamethasone may be used in human patients to treat in "all inflammatory, rheumatic and allergic conditions which respond to adrenocorticoid therapy..." The 1970 PDR specifically refers to potentially treating collagen diseases, skin diseases, allergies, inflammatory eye diseases, nonarticular musculoskeletal disorders, blood dyscrasias and "miscellaneous." The Examiner contends that "it has been known since the mid-1990s, that glucocorticoids effect their immunosuppression (sic) by reducing NF-kB activity by preventing NF-kB from binding to the appropriate sites on the DNA and/or by increasing the transcription, expression and/or release of IκB, thereby reducing the amount of activated NF-kB that can translocate into the nucleus." (Office Action at 36)

Examiner contends that a number of post-filing date published references (including Baldwin, Auphan, Scheinman I and II, Mukaida and Padgett) provide extrinsic evidence that any administration of dexamethasone would have necessarily reduced NF-kB activity and concomitant cytokine production. I respectfully disagree.

87. First, there is no showing in the 1970 PDR in connection with the different potential uses of glucocorticoids in patients of what, if anything, would necessarily induce NF-kB activity in cells in connection with any particular use, such that the induced NF-kB activity could be reduced as required by the claims. As the 1970 PDR lacks any such information, one skilled in the art would not read the 1970 PDR as teaching any of the methods claimed in the '516 patent. (Schematic 17 of **Exhibit 1**).

88. Second, these post-filing date published references (except for Baldwin and Padgett) all describe experiments conducted in various laboratory systems.[1] None of these references describe effects of using dexamethasone or other glucocorticoids in patients. From these experiments, the respective authors proposed various hypotheses in an attempt to explain the apparent effects of glucocorticoids they observed in their specific experimental systems. None of these later references provide data relating to clinical use of glucocorticoids in patients. Nor do they provide a reasonable basis from which one skilled in the art could conclude that administration of glucocorticoids under very different conditions than those described in the PDR demonstrates that dexamethasone, when used as described in the PDR necessarily resulted in reducing induced NF-kB activity, NF-kB-mediated intracellular signaling or any other NF-kB mediated effect in cells.

89. As Padgett acknowledges, while these different hypotheses (or "models" as Padgett refers to them) "provide insight into potential mechanisms" by which GC hormones can regulate

---

[1] The Examiner appears to refer to the Baldwin review article only for statements about what the other references describe. Baldwin does not report any primary data. Therefore, I will direct my comments to the actual data and discussion that these other references provide.

expression of certain immunologically related genes, "the story is not very clear." (Padgett at 446) For example, *in vitro* studies have reported contradictory results as to the effects of glucocorticoids on NF-kB binding activity, with some studies suggesting inhibitory effects, some studies demonstrating no effects, and some studies reporting enhancement of NF-kB binding activity. (Hart 2000 at 229). Moreover, as discussed below, other studies have been unable to confirm the different theories that Scheinman I and II, Auphan and Mukaida proposed, which suggested various potential mechanisms of action through which glucocorticoids might affect NF-kB.

90. The mechanisms by which glucocorticoids, including dexamethasone, mediate inflammatory responses are poorly understood, and as Padgett and these other papers discuss, substantial evidence demonstrates that glucocorticoids can act through a variety of mechanisms which are independent of NF-kB. (Padgett at 445, Han at 271, Hart at 224). For example, glucocorticoids function by binding in the cytoplasm to glucocorticoid receptors (GR), which then translocate to the nucleus as a glucocorticoid-GR complex and bind to target elements or glucocorticoid response elements (GREs) to function as direct enhancers or repressors of gene transcription. (Padgett at 445-46). As Padgett also notes, the glucocorticoid receptor "undoubtedly interferes with the function of other transcriptional regulators," such as activator protein-1 (AP-1) and NF-AT. (Padgett at 447, see also Han at 271, Hart at 224). Such evidence includes work from my laboratory, which demonstrated the ability of glucocorticoid receptor to prevent AP-1 dependent transcription through a mechanism likely involving protein-protein interactions between GR and Jun/AP-1. (Schule et al 1990, attached hereto as **Exhibit 16**.)

91. Moreover, none of the post-filing date references cited in the Office Action provides evidence that use of dexamethasone in patients as described in the 1970 PDR would

have necessarily reduced NF-kB activity and concomitant NF-kB mediated expression of any cytokine. As discussed below, in addition to poor understanding of their mechanisms of action, there is substantial contradictory data regarding the potential effects of glucocorticoids on transcription. To explain what necessarily occurred during prior use of dexamethasone as described in the 1970 PDR, a subsequent study would have to replicate the conditions of using dexamethasone the PDR describes. None of these studies do so. Scheinman II and Auphan both describe experiments that were conducted to examine the effect of dexamethasone on IkB expression in cultured cells. Neither study reproduces, nor attempts to reproduce the physiological conditions associated with administration of dexamethasone to patients described in the 1970 PDR. Further, neither study administered dexamethasone to cells in which NF-kB activity had first been induced by some external influence. Even if one assumed *arguendo* the 1970 PDR describe that a method in which NF-kB activity had first been induced, none of these later studies replicated such a method.

92. The data in Scheinman II were obtained primarily from experiments conducted with HeLa cells, a line of cancer cells having an abnormal genotype (with about twice the normal number of chromosomes). Even before induction with TNF-alpha, Scheinman observed high levels of NF-kB binding activity in the HeLa cells he used (Scheinman II at 284, Figure 1A), demonstrating that the pattern of NF-kB activity in the HeLa cells used by Scheinman does not reflect that found in normal cells. The data in Auphan reflect experiments conducted primarily with glucocorticoid receptor negative Jurkat cells stably transfected with an expression vector encoding rat GR (Auphan at 287-289, Fig. 2 through 4) or in other cell lines or mice treated with non-physiological inducers, such as ionomycin, TPA or anti-CD3 antibodies, which one would not encounter in patients (Auphan, Figures 1 and 3). Neither Scheinman II nor Auphan therefore

replicates any method of using dexamethasone that was described in the 1970 PDR. Moreover, in all the experiments described in Scheinman and Auphan, when used, dexamethasone was administered only before or simultaneously with any inducer. Therefore, neither of these studies describes the result of using dexamethasone in a method where it was used to reduce NF-kB activity that has first been induced by an external stimulus.

93. The data in Scheinman I was obtained primarily through a transfected cell system, which was engineered to overexpress an NF-kB component in the cells to allow the investigators to study potential interactions between the glucocorticoid receptor complex and NF-kB. (Scheinman I at 945) These interactions were assessed through transactivation of a reporter construct, which was also transfected into the cells. (See for example, Scheinman I at 945-47, Figs. 1 through 3). Activation of this reporter construct was driven by overexpression of p65 NF-kB subunit rather than by any external influence. (See for example, Scheinman I, Fig. 1). Significantly, none of these transactivation experiments describe the result of using dexamethasone in cells in which NF-kB had been induced by some external influence, or reflect a process in which NF-kB activity would be induced in any cell in the body.

94. Moreover, such engineered cells would not have existed in patients treated with dexamethasone as described in the 1970 PDR. The effect of dexamethasone on such engineered cells, in particular its effect on expression of any exogenous gene, would differ from its effect on any cell normally found in the body. Furthermore, in the single experiment conducted in untransfected (HeLa) cells, cells were pretreated with dexamethasone for 16 hours before administration of an inducer (TNF-α). (Scheinman at 949, Fig. 7). None of the experiments described in Schienman I indicate the effect of administering dexamethasone to cells in which NF-kB had been induced by some external influence.

95. Mukaida describes a series of experiments conducted to examine the potential mechanism of action of dexamethasone in preventing IL-8 expression in a human glioblastoma cell stimulated with IL-1. As in the references above, none of the experiments Mukaida describes attempts to reproduce the physiological conditions associated with administration of dexamethasone described in the 1970 PDR. In this regard, the PDR does not describe use of glucocorticoids to affect NF-kB. Further, none of these experiments addressed the effect of dexamethasone on cells in which NF-kB activity had been induced by some external influence, as dexamethasone was always administered concurrently with the inducing agent. Moreover, the authors' conclusion that "collectively, our results favor the assumption that the NF-kB site was responsible for IL-8 gene repression by dexamethasone," is inconsistent with the studies described in the Bourke reference discussed below. (Mukaida at 13294, Bourke at 2113, 2118). In summary, neither Mukaida nor any of the other post-filing data published references cited in the Office Action indicates that the administration dexamethasone as taught in the 1970 PDR would necessarily reduce induced NF-kB activity or NF-kB mediated-intracellular signaling.

96. In contrast to the post-filing date published reference cited by the Examiner, substantial evidence, which was not cited, including data as to the effect of glucocorticoids in patients, demonstrates that administration of glucocorticoids, in particular to treat inflammatory disorders, does not necessarily affect NF-kB activity. This evidence specifically demonstrates that the effects of glucocorticoid administration are not necessarily mediated through any of the potential mechanisms of action proposed by the models above or even by other potential mechanisms involving NF-kB. Instead, these studies indicate that glucocorticoids can exert anti-inflammatory effects in cells by mechanism(s) entirely independent of, and without affecting, NF-kB. Several such studies are summarized here.

97. Hart et al. 2000 attached as **Exhibit 17** examined the effect of glucocorticoid (fluticasone) administration on expression and DNA-binding activity of NF-kB in alveolar macrophages and bronchial biopsies from patients with asthma. While inhaled fluticasone decreased the inflammatory response in these asthma patients as assayed by several measures, "there was no decrease in NF-kB-DNA binding and activation." (Hart at 224). In fact, glucocorticoid treatment caused an increase in p65 expression in biopsy sections as determined by immunohistochemistry. Similarly, *in vitro* studies on A549 epithelial cells also showed that dexamethasone increased the expression of p65 when the cells were stimulated with IL-1β, suggesting that administration of glucocorticoid may cause increased transcription of NF-kB. (Hart at 224-225, 229-230). The authors concluded that "our data do not provide support for the actions of glucocorticoids occurring through the binding of its activated receptor to the transcription factor, NF-kB, because no reduction in the binding of NF-kB or its activation was apparent after 1 mo of treatment with a potent inhaled corticosteroid." (Hart 2000 at 229).

98. Bergman I (Bergman et al. Immunology 2004) and Bergman II (Bergman et al. Am. J. Resp. Biol. 2004) attached as **Exhibits 18** and **19** describe a series of studies conducted to examine the mechanism by which dexamethasone inhibited expression of granulocyte macrophage-colony-stimulating factor (GM-CSF) in primary human T-cells, human peripheral blood mononuclear cells and human T-cell lines (Jurkat and HUT78). GM-CSF is one of the proinflammatory cytokines that is activated in inflammatory disorders such as asthma, whose expression is regulated by NF-kB, as well as other transcription factors. (Bergman II at 555). NF-kB did not appear to mediate the inhibitory effect of dexamethasone on on GM-CSF expression. Specifically, although dexamethasone strongly inhibited GM-CSF release from cells stimulated by a combination of PMA and PHA, in reporter assays, dexamethasone had "no clear

effect" on NF-kB-dependent transcription (Bergman II at 561, Bergman I at 432-433, Fig. 2). These data indicated that dexamethasone likely exerted its inhibitory effect through some post-transcriptional/translational mechanism. (Bergman II at 555, 561-562).

99. Han et al. 2001 attached as **Exhibit 20** describes a series of experiments examining whether the ability of dexamethasone to suppress production of inflammatory cytokines in fibroblast-like rheumatoid synoviocytes (FLS) isolated from human subjects was mediated by an effect of dexamethasone on NF-kB. Stimulation of FLS cells with TNF-α induced NF-kB binding activity, as well as expression of two inflammatory cytokines believed to be involved in the pathophysiology of rheumatoid arthritis, IL-6 and IL-1. (Han at 268-269). Although simultaneous administration of dexamethasone suppressed the production of these cytokines, dexamethasone had no effect of DNA binding, and neither prevented translocation of NF-kB to the nucleus, nor induced synthesis of IkB-α. (Han at 267, 269-270, Figs. 1-4).

100. Bourke and Moynagh 1999 attached as **Exhibit 21** reports on a series of experiments using brain cell lines "strongly suggesting" that dexamethasone may exert antiinflamatory effects by mechanisms independent of NF-κB. (Bourke at 2113). These data demonstrated that pretreatment with dexamethasone inhibited expression of NF-κB regulated proteins IL-8, VCAM-1 and ICAM-1 in cells stimulated with IL-1. The authors found, however, that "the range of concentrations of dexamethasone that inhibited the expression of the proinflammatory molecules were completely ineffectual in affecting the activation of NF-κB." (Bourke at 2118). In particular, dexamethasone failed to prevent IL-1 from stimulating the nuclear translocation and DNA binding activity of NF-κB as determined by EMSA analysis. (Bourke at 2117-2118, Fig. 6) Moreover, dexamethasone also failed to affect IL-1 induced expression of an NF-κB-regulated reporter gene. Based on their data, the authors conclude that

"the scenario whereby glucocorticoids may produce immunosuppressive and anti-inflammatory effects in cells and NF-kB need not be involved in mediating the effects may be widespread and is not limited to a single cell type." (Bourke at 2118).

101. In summary, the fact that dexamethasone (or any other glucocorticoid) is administered to cells does not indicate that the administration of that glucocorticoid necessarily reduced induced NF-kB activity or NF-kB mediated-intracellular signaling. In this regard, the 1970 PDR provides no actual data relating to use of glucocorticoids, and no evidence that in the conditions the PDR indicates could be potentially treated with glucocorticoids, cells would have been acted on by a particular external influence that would have necessarily induced NF-kB activity or any NF-kB mediated effects. Furthermore, the 1970 PDR also provides no evidence that administration of glucocorticoids would have reduced induced NF-kB activity, would have affected expression of NF-kB regulated genes, or would have influenced any other NF-kB mediated effect in the cell.

(iii) Inherent anticipation rejection based on pages 1466-1496 of Goodman and Gilman (1980) relating to production and release of endogenous glucocorticoids

102. I understand that the Examiner has also rejected claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31-32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97 as anticipated by pages 1466 to 1496 of Goodman and Gilman (1980), and explained by various cited non-prior art references. (Office Action at p. 55). I have reviewed the cited pages from Goodman and Gilman, the cited non-prior art references and the above claims. The Examiner contends that Goodman and Gilman teaches "in response to various environmental stressors," that cortisol and other endogenous glucocorticoids are produced and released, which, as explained by the cited non-prior art references, would necessarily reduce NF-kB activity and expression of cytokines

whose transcription is regulated by NF-kB, and would therefore have carried out the above claims. (Office Action at 55). I respectfully disagree.

103. First, as discussed above, the claims are directed to methods for modifying the naturally occurring response to such inducing stimuli and reducing the harmful effect of such external influences by "reducing NF-kB activity." These goals are achieved by reducing the ability of NF-kB to act as a messenger inside the cell regulating specific results recited in the claims, such as reducing NF-kB mediated gene expression. When read in light of the teachings of the patent disclosure, one skilled in the art would have understood the claims to require affirmative, manipulative steps, calculated to achieve such specific results. One skilled in the art, therefore, would not have understood the natural production and release of endogenous glucocorticoids as carrying out any of the '516 patent claims.

104. Second, there is no showing in Goodman and Gilman (1980) in connection with the widely variable "environmental stressors" referred to ("agonal state, severe infections, surgery, parturition, cold, exercise and emotional stress"), of what, if anything, would necessarily induce NF-kB activity in cells in connection with any particular stressor, such that the induced NF-kB activity could be reduced as required by the claims. As it lacks any such information, even if one read the claims to encompass natural production of endogenous glucocorticoids, one would not read the Goodman and Gilman (1980) as describing any of the claimed methods. The Examiner refers to "hypersensitivity reactions" but provides no evidence, nor is there any reason to believe, that the different environmental stressors that the Examiner contends would cause production of endogenous glucocorticoids would necessarily be associated with "hypersensitivity reactions." (Office Action, at 56.)

105. Third, the allegedly anticipating pre-April 21, 1989 art the Examiner relies on describes cortisol. The later references the Examiner relies on regarding effects on NF-kB all describe various experiments using the synthetic steroid dexamethasone, which is a different compound. One cannot assume that the effects of different glucocorticoids and steroids are necessarily the same, as is evident from the fact that numerous, different steroid drugs have been developed, and have different indications. Further, as Padgett indicates, glucocorticoids mediate effects through at least two different receptors, the glucocorticoid receptor and the mineralocorticoid receptor. (Padgett at 445). As Goodman and Gilman indicate, and as evidenced from numerous later publications, the spectrum of biological effects of different glucocorticoids can vary tremendously at least in part due to the different interactions of each distinct glucocorticoid with each of these receptors, and the specific pattern of receptor expression on any individual cell. (For example, see Goodman and Gilman at 1473). Although the Examiner asserts that Padgett provides analogous "NF-kB inhibiting evidence" as that in Baldwin I, Auphan, Scheinman I/II and the Mukaida documents regarding dexamethasone, the Padgett review provides no primary data, and does not discuss any experiment which examined the effect of cortisol (or any endogenous glucocorticoid) on NF-kB activity. The Examiner has no basis in fact for assuming that cortisol would necessarily affect NF-kB in the same way under any particular experimental scenario.

106. Lastly, even if one were to erroneously assume that one could equate the effect of cortisol with dexamethasone, for all the same reasons as explained above regarding use of dexamethasone, the fact that cells were exposed to cortisol produced and released naturally in the body does not indicate that such exposure necessarily reduced induced NF-kB activity or NF-kB

mediated-intracellular signaling. For all these reasons, pages 1466-1496 from Goodman and Gilman cannot anticipate any of the above claims.

VI. Calcitriol

107. Calcitriol (1,25-$(OH)_2D_3$) is the active metabolite of vitamin D3 (cholecalciferol). It is widely thought that the biological effects of calcitriol are mediated through its interaction with a high affinity nuclear receptor known as the vitamin D receptor (VDR). (Alroy 1995). The receptor selectively associates with recognition sequences generally in the promoter region of target genes, and thereby acts to either positively or negatively regulate transcription of those genes. (Alroy at 5780-90). Notably, calcitriol appears to inhibit transcription through the VDR receptor by acting directly as a repressor for transcription, but in some genes, notably IL-2, it also appears to inhibit transcription by interacting with the region in the promoter that comprises the binding site for NFAT and preventing NFAT induction. (Alroy at 5790).

A. Inherent Anticipation Rejection Based on Pre-April 21, 1989 References Relating to Calcitriol (Tsoukas, Manolagas, Lemire I and II, Rigby I and II)

108. I understand that the Examiner has rejected claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31-32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97 as anticipated by Tsoukas, Lemire I, Lemire II, Rigby I, Rigby II or Manolagas. I have reviewed each of these references and the above claims. The Examiner contends that each of these references teaches administration of calcitirol to humans, and also reports the study of calcitriol in various human cell cultures, including human Jurkat (T-cell line), leukemic cells, and human peripheral blood monocyte ("PBM") cells, including Tsoukas (PBM), Manolagas (PBM), Lemire I, Lemiore II, Rigby I, and Rigby II. (Office Action at 31). The Examiner contends further that the cited extrinsic evidence (post-filing date published Yu 1995, and statements in paragraphs 7 through

24 of the Declaration of Dr. Manolagas) show that administration of calcitriol as described in these references would have necessarily reduced NF-kB activity and also expression of genes whose transcription is regulated by NF-kB activity. The Examiner therefore contends that each of the primary references inherently anticipates the above claims. I have reviewed Yu 1995 and the Declaration of Dr. Manolagas, and I respectfully disagree.

109. As an initial matter, the Examiner should note that the cited publications all describe experiments conducted in various types of cultured cells. None of these publications describe methods for using calcitriol in humans. Therefore, this declaration will only address the contentions made in the Office Action regarding use of calcitriol in cultured cell systems, as described in the cited primary publications.

110. In particular, the Examiner relies on six primary publications: Tsoukas and Manolagas, describing work done in Dr. Manolagas laboratory, and four other publications by others (Lemire I and II, Rigby I and II). The Examiner relies on these studies as evidence that administration of calcitriol in "PBM" cells reduced production of the cytokine IL-2, which the Examiner argues is regulated at least in part by NF-kB. (Office Action at 30-31). The Examiner contends that Yu et al. carried out experiments "under the same conditions utilized in Tsoukas and Manolagas" and therefore replicated the conditions of using calcitriol in the primary references. (Office Action at 31.) The Examiner argues that Yu's data "that calcitriol reduced NF-kB activity as well as NF-kB-regulated gene expression," therefore demonstrate that administration of calcitriol as described in the pre-April 21, 1989 references would have "necessarily and inherently" reduced NF-kB in induced human cells (PBM/Jurkat) to reduce IL-2 expression, and "necessarily and inherently" practiced the above claims. I respectfully disagree.

111. Each of the above claims requires "reducing NF-kB activity." As described above, I understand all the above claims are directed to methods carried out on cells in which NF-kB activity has first been induced by some inducing external stimulus. In this regard, the Examiner erroneously states the primary references describe the use of calcitriol in induced human cells. Tsoukas 1984 describes a series of experiments investigating the effect of calcitriol on proliferation and production of IL-2 protein (as measured indirectly by IL-2 activity) in PBM cells simultaneously stimulated with PHA, not first induced with PHA. Manolagas 1986 reports further experiments investigating the effect of calcitriol on proliferation and IL-2 activity in PBM cells, in terms of its calcium sensitivity. None of these experiments were conducted by administering calcitriol by itself to induced cells. Therefore, none of the methods of using calcitriol by itself that Tsoukas or Manolagas describe could have involved reducing induced NF-kB activity.

112. Lemire I and II describe experiments investigating the effect of calcitriol on DNA synthesis and IgG production in PBM cells. In Lemire I, calcitriol treated cells were simultaneously stimulated with a number of different agents, either PHA, pokeweed mitogen (PWA) or dermatophyton-O. In Lemire II, cells were either pretreated or simultaneously treated with calcitriol and a number of different agents, either PHA, PWA, or con A. The only specific data reported by either Lemire I or II from PHA treated cells relates to the effect of calcitriol on DNA synthesis (Lemire I at 659, Fig. 2; Lemire II at 3033, Table I, Fig. 1). Again, none of these experiments were conducted by administering calcitriol to cells in which NF-kB activity had first been induced. Instead, these experiments were conducted by simultaneously treating cultures with calcitriol and various agents. Therefore, none of the methods of using calcitriol that Lemire I and II describe could have reduced induced NF-kB activity as recited in the claims.

113. Rigby II describes experiments investigating the effect of calcitriol on mitogenesis (cell cycle distribution and DNA synthesis), cell surface levels of IL2 receptors and transferrin receptors in PBM cells. In Rigby II, none of the experiments were conducted by administering calcitriol to induced cells. Instead, in these experiments, cells were always stimulated with PHA in the presence of calcitriol. None of these experiments could therefore have used calcitriol to reduce induced NF-kB activity.

114. Rigby I describes experiments investigating the effect of calcitriol on mitogenesis and production of IL2 (by a bioactivity assay) in PBM cells. In certain of the experiments, calcitriol was administered to cells at various time points after stimulation with PHA. (Rigby I at 1453, Figs. 2 and 4). Of the calcitriol pre-April 21, 1989 art cited, Rigby is the only study to describe any instances of administering calcitriol to cells after stimulation with PHA. To the extent the Examiner relied on these particular experiments to contend that, based on Yu, such use of calcitriol would have necessarily reduced NF-kB activity such that IL-2 expression was reduced, substantial evidence demonstrates that there is no reasonable factual basis for such an inference. This evidence, summarized below, includes significant differences in protocols between Yu 1995 and earlier studies, lack of any correlation between the purported kinetics of the effect of calcitriol on NF-kB binding and the effect of calcitriol on production of IL-2 in earlier studies, evidence that calcitriol inhibits IL-2 expression through NFAT, and not NF-kB, as well as methodological deficiencies which bring into question the purported observations Yu 1995 makes regarding NF-kB.

(i) Yu 1995 does not reproduce the pre-April 21, 1989 art

115. One of skill in the art would understand that Yu fails to reproduce the conditions for using calcitriol described by the studies described in the primary cited reference, and

therefore could not, and does not provide an explanation for what occurred in these earlier studies. Among other differences, there are substantial differences between the "PBM" cells that Yu used and those used in all the cited primary studies from the 1980s, including Rigby I. As explained below, these differences would have significantly impacted the effects calcitriol would have had. Therefore, even assuming that Yu showed that calcitriol affected NF-kB in some way (which in my opinion it does not), there is no basis for inferring that any of the results Yu observed explain the reported prior use with a significantly different cell population. In this regard, one cannot properly state that the experiments in Yu were conducted "under the same conditions utilized in Tsoukas and Manolagas," or in any of the other publications from the 1980s cited by the Examiner.

116. As discussed above, the term "PBM cells" would not be understood by one skilled in the art to refer to a single, defined cell type. Such cell cultures comprise a highly heterogeneous cell population comprised of numerous immune cell types isolated from patients blood samples, including T and B-lymphocytes, NK (natural killer) cells, monocytes and macrophages. (See for example, David et al. Blood (1998)). There is substantial variability in PBM cells, depending on the protocol and conditions of isolation. Thus, careful attention to details would be required to replicate conditions of earlier studies. Rigby I, as well the other cited pre-April 21, 1989 art, including Tsoukas and Manolagas, used cells isolated from blood samples through different modifications of Ficoll-Hypaque discontinuous gradient centrifugation. (See for example, Rigby I at 1452). Instead, the experiments with "PBMCs" described in Yu 1995 relied on a new protocol, described in Yu 1991 (reference 15), for further fractionating this highly heterogeneous cell population into plastic-adherent and non-adherent cells, and using only the plastic non-adherent cell population. (Yu 1995 at 10991, Yu 1991 at

7589). None of the cited primary reference isolated and used a plastic-non-adherent cell population.

117. Significantly, "the adherence step was performed to eliminate the majority of the adherent monocytes" which constitutively express the vitamin D receptor (VDR), unlike the remaining non-adherent lymphocytes, where VDR expression varies depending on the state of the cell. (Yu 1991 at 7588-89) attached as **Exhibit 22**. Especially in regard to evaluating the effects of calcitriol, removing the adherent (and calcitriol responsive) monocytes from the cell population would most likely have a substantial effect on the overall effect of calcitriol in the cell culture. In view of this important difference between Yu 1995 and the cited primary reference, one skilled in the art could not assume or infer that any of the purported effects on NF-kB reported by Yu 1995 on "PBMCs" (even if true) would have necessarily been observed in any of the earlier studies with "PBM" cells.

(ii) There is no evidence that NF-kB mediated any affect of calcitriol observed in the cited primary references

118. Further, none of the studies reputted in the primary reference provide evidence that calcitriol affected any process in the cell that would have been mediated through an effect of calcitriol on NF-kB. Most notably, there is no correlation between the time course for the processes measured in the earlier studies, such as IL-2 production and proliferation, and the purported inhibitory effects of calcitriol on NF-kB Yu described in 1995. In particular, the authors report that substantial induction of p50 NF-kB expression occurred only 72 hours after treatment of PBMCs with PHA, with virtually no induction detectable at 24 hours (Yu 1995 at 10991, Fig. 1)(A more legible copy of Yu 1995 is attached as **Exhibit 23**). In contrast, earlier studies observed that IL2 production peaked no later than 24 hours after PHA treatment. In other

words, from these data, there is no basis for concluding that IL2 production was linked in any way to induction of NF-kB activity.

119. In fact, substantially more comprehensive analyses of the IL-2 promoter in other studies has demonstrated that repression by calcitriol of IL-2 expression, as well as aspects of its immunosuppressive activity in T cells likely occurs through an NF-kB independent mechanism involving NFAT-driven transcription as a target. (Alroy et al 1995, Takeuchi et al. 1998 attached hereto as **Exhibits 24** and **25**). For example, Alroy et al. in PHA/PMA stimulated Jurkat cells, found when activated by calcitriol, the vitamin D3 receptor directly repressed the ability of NFAT to activate transcription of the IL-2 gene by interfering with assembly and binding of an NFAT/AP1 complex to the IL2 promoter, and thereby reduced IL2 gene expression. (Alroy at 6796, see also Alroy Figs. 3, 5 and 8). Similarly, Takeuchi found that calcitriol treatment of activated T cells "resulted in inhibition of NFAT complex formation" to the human IL-2 promoter NFAT site, as well as diminished NFAT-driven transcription of a reporter gene in calcitriol-treated Jurkat cells. (Takeuchi at 210, 215, Figs. 1 and 6). Notably, Takeuchi "found no significant effect of [calcitriol] on NFkB binding activity under T cell activation conditions [ionomycin and PMA] used here." (Takeuchi at 215).

120. Similarly, there is no evidence that NF-kB mediated any of the other effects of calcitriol that were investigated in the earlier studies described in the primary references. Calcitriol inhibited proliferation of PBMCs only when it was added during the first 24 hours data suggesting "that the antiproliferative effect of $1,25\text{-}(OH)_2D_3$ is not the result of its effects on NF-kB." (Yu 1995 at 10994). These data therefore fail to demonstrate that the apparent inhibitory effects of calcitriol on NF-kB had any functional significance. Recognizing the inconclusive data, Yu indicates that the relevance of the apparent inhibitory effects they observed calcitriol to

have on NF-kB in their study to any of its immunoregulatory properties "is a matter of conjecture," in other words, mere speculation. (Yu 1995 at 10994). Such speculation cannot reasonably support a conclusion that the effect of calcitriol on IL-2 production or cell proliferation provides evidence that use of calcitriol in any study described in any of the primary reference necessarily reduced NF-kB activity and consequent expression of NF-kB regulated proteins.

121. Additionally, the Examiner refers to experiments conducted in Jurkat cells transfected with a CAT reporter construct, in which the CAT gene was placed under control of a quadruple repeat of an NF-kB consensus sequence. (Yu 1995 at 10993, Fig. 6). The Examiner argues that these data prove that administration of calcitriol to PBM cells in the pre-April 21, 1989 studies would have resulted in reduced NF-kB gene expression. (Office Action at 32). I respectfully disagree.

122. The CAT reporter experiments do not reproduce any use of calcitriol described in the cited primary reference. First, the CAT reporter assays do not replicate use of calcitriol in induced cells as required by the claims, because the cells were pretreated with calcitriol for 40 hours before being stimulated with PHA. More significantly, the transfected Jurkat cells used by Yu differ significantly from the PBM cells and Jurkat cells used in the prior studies. In particular, the artificial (quadruple repeat) NF-kB binding site, on which the reporter construct was based, is not found in any endogenous gene, and would not have been present in any of the cells used in the earlier studies. There is no basis for assuming that the response of this reporter construct reflects the regulation of any endogenous gene. These CAT data therefore provide no basis for assuming that calcitriol, as used in the prior studies, would have necessarily affected NF-kB mediated gene expression.

(iii) Yu 1995 does not substantiate that calcitriol affects NF-kB binding activity in induced cells

123. Additionally, the Examiner refers to EMSA assays described in Yu as evidence that calcitriol reduced NF-kB activity. The Examiner notes that Yu observed that calcitriol administration "caused a significant reduction in the DNA-protein complex, as evidenced by the decrease in the intensity of this band (lane 4)" (Office Action at 31, Yu at 10993, Fig. 5). Although Yu interprets these data to indicate that calcitriol decreases NF-kB-DNA binding activity, I find that conclusion questionable in view of the inconsistencies and methodological flaws underlying these EMSA data.

124. In particular, the assumption that the bound complex represents only NF-kB binding activity is particularly questionable. Based on my experience with EMSA assays, including NF-kB EMSA assays, one would not achieve significant competition, as was observed here, with only a 5-fold excess of unlabeled competitor. (Yu Fig. 5, lane 5). Furthermore, Yu observed that competition with a "non-specific" oligonucleotide containing an AP-1 binding motif actually increased binding to the labeled EMSA probe. (Yu Fig. 5, lane 7). One of skill in the art would not expect such a sequence to affect binding to the NF-kB site. Such results indicate a likely problem with specificity, such that further appropriate controls, such as antibody controls or mutational controls, would be required to substantiate that the apparent inhibition represented an effect on NF-kB binding, and not the binding of some other factor, to the probe. Additionally, even if these data were to be confirmed, it should be noted that the EMSA assay does not directly assess the effect of calcitriol on induced cells, as the cells studied by Yu were simultaneously treated with both calcitriol and PHA, i.e. not first induced by an external stimulus.

B. Studies show that calcitriol treatment can increase NF-kB activity

125. In contrast to the Examiner's assumption that administration of calcitriol would necessarily reduce NF-kB activity, studies have shown that calcitriol in fact induces NF-kB activity, i.e. stimulates induction of NF-kB by external influences (See, e.g., Schematic 14 of **Exhibit 1**). In particular, a number of studies that Dr. Manolagas relies on in his declaration purportedly proving that calcitriol necessarily reduces NF-kB activity instead demonstrate the opposite. For example, both Adams et al. (2004) and Berry et al. (2002) (Manolagas Declaration ¶23) observed that treatment with calcitriol resulted in increased NF-kB activity under the conditions studied. Adams reports that calcitriol alone stimulated NF-kB activity in C3H10T1/2 cells. (Adams at 2948 attached as **Exhibit 26**). Berry reports that calcitriol increased NF-kB activity in a TPA-stimulated promyeloytic leukemia cells by stimulating phosphorylation and degradation of IkB. (Berry at 183 **Exhibit 27**). Such studies provide further evidence that the Examiner is incorrect in his assumption that calcitriol administration to cells necessarily reduces NF-kB activity. Therefore, the use of calcitriol as described in the prior studies is not the case of a method recited in any of the above claims.

VII. Antibiotics

A. Inherent Anticipation Rejection Based on Portions from the 1970 PDR

(i) 1970 PDR

126. I understand the Examiner has rejected claims 1-2, 5, 6, 8-10, 12-18, 20-21, 25-27, 29-32, 36-41, 53-54, 58-65, 69-76, 80, 82, 84, 87-89, 93-100, 104-105, 119-120, 124-129, 133-140, 144-150, 154-160, 164-168, 172-178 and 182-186 as anticipated based on portions of the 1970 PDR, as explained by various post-filing date published references cited in the Office Action, and the Declaration of Dr. Manolagas. I have reviewed these portions of the 1970 PDR,

the cited post published references, the Manolagas declaration and the above claims. The Examiner argues that the 1970 PDR teaches administration of various antibiotics, such as erythromycin, gentamicin and tetracycline to kill bacteria. The Examiner contends that such use of antibiotics would reduce production of LPS from the bacteria, which would therefore inherently reduce LPS-induced and NF-kB regulated cytokine production, and therefore inherently practice the above claims. I respectfully disagree.

127. Each of the above claims requires "reducing NF-kB activity." As previously discussed, I understand all the above claims are directed to methods carried out on cells in which NF-kB activity has first been induced by some inducing external stimulus, in other words a eukaryotic cell in which NF-kB activity is activated by the presence of an external influence. Antibiotics have no effect on any of the segments of the NF-kB pathway as illustrated in Schematic 10 of **Exhibit 1**. As indicated by the 1970 PDR, and acknowledged by the Examiner, antibiotics either kill or interfere with growth of bacterial cells. Antibiotics do not act "by blocking LPS that reaches the cell," as the Examiner erroneously alleged on page 42 of the Office Action. As also illustrated on Schematic 10, antibiotics neither act on the mammalian cell, nor do they act to interfere with the induction by the external influence to which the Examiner refers, i.e. LPS. One skilled in the art seeking to practice any of the above claims would therefore never contemplate using antibiotics to "reduce NF-kB activity." Even if one viewed the external stimulus as the bacterial infection, antibiotics act on the bacteria and do not act on any of the 6 segments of NF-kB pathway.

128. While at least for the above reasons, use of antibiotics would not practice any of the above claims, the Examiner should also note that the factual premise that use of antibiotics to treat bacterial infections would necessarily reduce LPS levels is incorrect. In this regard, the

Manolagas declaration indicates that one possible (but not necessary) outcome of antibiotic treatment is to reduce LPS levels, contingent on completely resolving a bacterial infection. (Manolagas Decl. ¶¶ 38-39.) However, as acknowledged by the PDR there are several common reasons why antibiotic treatment fails to do so. For example, where certain of the bacterial cells are resistant to a particular antibiotic, antibiotic treatment can actually increase growth of any resistant bacterial strains present in the treated patient. Moreover, rather than having bactericidal effects, antibiotics often have bacteriostatic effects which do not result in killing bacteria. (1970 PDR at 880, 1167, 1309, 1310, 1379). Thus, the bacteria with LPS remains and continues to induce NF-κB. Finally, antibiotics often have little effect on enteric bacteria, which include gram-negative bacteria that carry LPS on their surface.

129. As evidence that antibiotic treatment would reduce LPS levels, Dr. Manolagas specifically refers to only one paper. (Galdiero (2001), Manolagas Declaration ¶¶ 37-39). Galdiero however, neither mentions antibiotics, nor does the paper provide any information relating to the effect of LPS in any human patient. Indeed, Galdiero indicates that in cultured cells, even extremely low levels of LPS, such as 10ng/ml were sufficient to induce release of cytokines from the cells. Dr. Manolagas' assumption that in a patient, LPS levels to below 1ug/ml would no longer stimulate NF-κB-mediated cytokine production therefore has no support in Galdiero, which indicates that 100-fold lower levels of LPS (0.01ug/ml) induced expression of such genes in cultured cells.

130. Numerous studies, in fact, have substantiated that antibiotic treatment can disrupt bacterial membranes, which by releasing and solubilizing LPS, can be expected to increase circulating levels of LPS in the body. See for example, Lepper et al., *Clinical Implications of Antibiotic-Induced Endotoxin Release in Septic Shock*, Int. Care Med. 28:824-833 (2002)

(**Exhibit 28**); Shenep and Mogan, *Kinetics of Endotoxin Release During Antibiotic Therapy for Experimental Gram-Negative Bacterial Sepsis*, J. Inf. Diseases, 150:380-388 (1984) (**Exhibit 29**); Hurley et al., *Antibiotic Release of Endotoxin in Chronically Bacteuriuric Patients*, Antimicrob. Agents Chemother. 351: 2388-2394 (1991) (**Exhibit 30**); Dofferhoff et al., *Effects of Different Types and Combinations of Antimicrobial Agents on Endotoxin Release from Gram-negative Bacteria: An In Vitro and In-Vivo Study*, Scand. J. Infect. Dis. 23:745-754 (1991) (**Exhibit 31**); Mustafa et al., *Increased Endotoxin and Interleukin-1 Beta Concentrations in Cerebrospinal Fluid of Infants with Coliform Meningitis and Ventriculitis Association With Intraventricular Gentamicin Therapy*, J. Infect. Dis. 160:891-5 (1989) (**Exhibit 32**). LPS released and solubilized from bacterial membranes may have substantially increased biological activity. Lepper 2002 at 825-826. Leeson and Morrison, *Induction of Proinflammatory Responses in Human Monocytes By Particulate and Soluble Forms of Lipopolysaccharide*, Shock 2:235-245 (1994) (**Exhibit 33**). In summary, such evidence demonstrates that when used to treat gram-negative infections, by inhibiting bacterial growth or by killing bacteria, antibiotics are likely to increase LPS levels, and further stimulate activation of NF-kB in the cell.

131. Finally, as discussed above, claims 8-9, 75-80, 82, 84, 88-98, are further directed to methods carried out on cells in which the "external influences induce NF-kB mediated intracellular signaling." These claims further require the methods to be carried out on cells in which the induction is maintained by the presence of the external influence. Killing bacteria, even if doing so could also remove the LPS, cannot practice any of the claims which require maintained induction by the external influence.

VIII. Red Wine

A. Inherent Anticipation Rejection Based on St Leger, Dobrilla or Jones

132. I understand that the Examiner has rejected claims 1-2, 6, 8, 9, 20-21, 25-27, 29-32, 36-41, 64-65, 69-76, 80, 82, 84, 87-89 and 93-98 based on excerpts from the King James Version of the Bible (portions), St Leger (1979), Dobrilla (1984) or Jones (1987), and alleged explained by post-filing date published reference Blanco-Colio 2000, Holmes-McNary (2000) and Manna (2000). I have reviewed each of these references and the claims. The Examiner appears to rely on the excerpt from the Bible simply for the proposition that red wine has been consumed for many years. The Examiner contends that St. Leger, Dobrilla and Jones describe studies in which there was "substantial" consumption of red wine along with "fatty foods." The Examiner argues that the post-filing date references explain that consumption of even a moderate amount of red wine with fatty food as purportedly described in the pre-April 21, 1989 references would have necessarily reduced NF-kB activity induced by the fat content in the food, as well as concomitant NF-kB mediated gene expression. (Office Action at 46-47). I respectfully disagree.

133. Each of the above claims requires "reducing NF-kB activity." As described above, I understand all the above claims are directed to methods carried out on cells in which NF-kB activity has first been induced by some inducing external stimulus. There is no showing or description in any of these of primary cited publications as to what, if anything, would have acted on a cell to induce NF-kB activity in any subject consuming red wine, let alone necessarily have done so. In this regard, these primary references also provide no information as to what agent, if any, would have necessarily acted on cells to reduce NF-kB activity in subjects consuming red wine. "Red wine" as a chemical mixture would not be found *per se* in the body after it is consumed and therefore could not act *per se* on cells (Schematic 15 of **Exhibit 1**). Red

wine is a generic term covering many different mixtures having in common the characteristic of being of red color. Each such mixture has many variable constituents, which these pre-April 21, 1989 studies never identified or analyzed. Without any description of what, if anything, would have induced NF-kB activity in cells, or what, if anything, would have acted to reduce NF-kB activity in cells, one skilled in the art would not read any of the primary references as describing any of the claimed methods.

134. St. Leger (1979) is simply a statistical analysis of mortality from cardiovascular disease in developed countries in which a negative association was observed between death from heart disease and consumption of wine. The study does not provide any details as to the type of food or red wine consumed by any subject, or describe any obvious inducing stimuli that would act on a cell. Notably, this epidemiological analysis even failed to suggest that consumption of red wine was advantageous compared to any other wine. (St Leger at 1020). In that regard, one could not even read St Leger as teaching one to specifically use red wine for any purpose.

135. Dobrilla (1984) describes a clinical study conducted to evaluate the effect of cimetidine and ranatadine on ethanol metabolism. Dobrilla does not provide any details as to the type of food or red wine consumed by any subject in the study, other than to note that all subjects before being tested had consumed a "standard weight ham sandwich."

136. Jones (1987) describes a study in which methanol elimination half-life was measured in four subjects who in the course of two hours, consumed an intoxicating amount of red wine (up to two standard 750ml bottles). Jones does not provide any details as to the type of food or red wine consumed by any subject other than that the red wine used in the study

contained substantial amounts (100mg/l) of methanol and that while drinking, the subjects ate some amount of potato chips and cheese.

137. Despite the fact that these primary references fail to describe any aspects of the claims, the Examiner contends that post-filing date published Blanco-Colio provides extrinsic evidence that St. Leger, Dobrilla and Jones necessarily involved use of the method claimed in the subject claims. I respectfully disagree. In particular, I disagree that these later references provide any factual basis for the notion that NF-kB would have been necessarily induced, or that any induced NF-kB activity would have been necessarily reduced in PBM cells of subjects consuming red wine. As explained below, such a conclusion could not be based on anything more than mere speculation. Further, neither Blanco-Colio nor anyone else would be readily able to replicate the experiments of the earlier studies because the type of red wine was not specified in those earlier studies.

138. The Examiner contends that the Blanco-Colio study explains that triglyceride lipoproteins would have necessarily acted on cells to induce NF-kB activity in virtually any person who had consumed "fatty food." Blanco-Colio reports that among the 16 subjects studied, a fat enriched breakfast increased blood levels of certain lipoproteins (total triglycerides and chylomycrons) but not others (VLDL). Even if the Blanco-Colio's interpretation of their NF-kB EMSA data were correct (and as I explain below why their interpretation is likely incorrect), there certainly is no evidence in the Blanco-Colio study identifying certain triglyceride lipoproteins as agents that had acted on cells to induce NF-kB activity. (Blanco-Colio at 1022). Blanco-Colio provides no direct evidence for this position.

Meichle reports experiments conducted with Jurkat cells transfected with two different CAT reporter constructs, each of which are described as containing a CAT gene and an enhancer with an NF-kB binding site. (Meichle at 8340). However, none of the experiments with these constructs, which are reported in Fig. 1, used any PK inhibitor. (Meichle at 8341, Fig. 3). Meichle provides no evidence whether PK inhibitors could prevent any agent from inducing expression of these constructs. Additionally, Meichle provides no data as to whether PK inhibitors affected expression of any endogenous gene. Therefore, one would not read Meichle as either describing or suggesting any use of PK inhibitors to reduce or NF-kB-mediated gene transcription by reducing NF-kB activity.

151. Finally, in the EMSA assays (Figs. 2 and 3), Meichle employed a 29 base pair oligonucleotide corresponding to a portion of the HIV enhancer. As discussed above, studies have documented that this sequence binds not only NF-kB but also other transcription factors including factors in the NFAT transcription factor family. To substantiate that the most slowly migrating complex observed in the EMSA assay corresponded to NF-kB, it would have been necessary to determine whether mutation of the putative NF-kB binding site abrogated binding. Without this control, the data Meichle provides are insufficient to support the authors' conclusion that PK inhibitors were able to reduce NF-kB binding activity. In summary, Meichle fails to provide any data that would have led one of ordinary skill in the art to reasonably expect that one could successfully use PK inhibitors to modify any NF-kB mediated effects or NF-kB mediated gene expression by reducing NF-kB activity.

139. At best, the authors imply that there was some correlation between blood levels of certain lipoproteins and NF-kB binding activity. (Blanco-Colio at 1024). However, in view of their data, that correlation is highly questionable, in particular when one examines data from subjects who had not consumed any red wine. (Blanco-Colio at 1022-23, Figs. 1 and 2). For example, Blanco-Colio purports to show a maximal increased NF-kB binding activity in PBM cells 9 hours after the subjects consumed breakfast. (Blanco-Colio at 1023, Fig. 2C). However, by 9 hours, the total triglyceride blood levels in those subjects had dropped to below baseline levels. (Blanco-Colio at 1022, Fig. 1A). Such a questionable correlation is far from sufficient to establish that triglycerides acted to induce NF-kB activity either in Blanco-Colio let alone, in any of the subjects in either the Dobrilla or Jones studies, which provide no information on triglyceride levels or NF-kB activity.

140. Moreover, I question the Blanco-Colio authors' interpretation of their EMSA data as demonstrating that NF-kB binding was reduced in subjects who consumed red wine along with breakfast. Notably, addition of anti-p65 NF-kB antibody to nuclear extracts from PBM cells caused a supershift of the lower portion of the major bound complex, indicating that the lower portion of the complex comprised bound p65 protein. (Blanco-Colio at 1024, Fig. 3, lane 4). These data in Fig. 3 strongly suggest that the lower unlabeled band in Fig. 2 may also correspond to NF-kB (p65) binding activity. (Blanco-Colio at 1023, Fig. 2, panels A and B). Notably, the authors failed to identify the unlabeled complex, and provide no explanation for why they did not interpret this band as corresponding to NF-kB. Significantly, if one were to assume this to be correct, as the data suggest, Fig. 2 would not support the authors' conclusion that NF-kB activity was reduced in subjects who had consumed red wine with breakfast.

141. The Examiner also refers to two post published references reporting various experiments on the effects of chemically pure unconjugated form of resveratrol, *trans*-resveratrol (3,5,4'-trihodroxy-*trans*-stilbene, or alternatively, *trans*-3,4',5-trihydroxystilbene), in cultured cells. (Holmes-McNary at 3477; Manna at 6509, 6511) The Examiner, however, does not refer to any pre-April 21, 1989 references describing use of chemically pure *trans*-resveratrol. Moreover, neither Holmes-McNary, nor Manna conducted any experiments with red wine. All their data were obtained using a chemically pure *trans*-resveratrol. In this regard, neither Holmes-McNary, nor Manna reproduced the use of red wine in Dobrilla or Jones and therefore cannot explain or confirm what occurred in those earlier studies

142. The Examiner contends that nevertheless, based on Holmes-McNary and Manna, that red wine as used in Dobrilla and Jones would necessarily have the same effects on NF-kB as Holmes-McNary and Manna purportedly using pure *trans*-resveratrol in cultured cells. I respectfully disagree. Such a theory is premised on a number of unsupported and erroneous assumptions. Among these, this theory assumes that after consuming red wine, resveratrol in any particular variety of red wine, if any, remains bioavailable and in the same chemical form as *trans*-resveratrol used in the *in vitro* experiments of Holmes-McNary or Manna. Several clinical studies have demonstrated that when tested, these assumptions prove to be incorrect.

143. Vitaglione et al. attached as **Exhibit 34** conducted a clinical study verify if free *trans*-resveratrol or alternatively metabolized forms, could be found in human serum after consuming half (or more) of a standard bottle of red wine (300-600ml) in or without association with a meal. (Vitaglione at 496). The study involved three separate experiments with 25 volunteer subjects who consumed red wine and different meals with varying lipid content, including a "fat meal." *Id.* Blood samples were taken at different intervals and the serum

samples obtained were analyzed by three different HPLC techniques to quantify the levels of free *trans*-resveratrol and metabolites. *Id.*

144. Strikingly, resveratrol was never found in any form at any time in 14 of the 25 subjects tested. (Vitaglione at 502). Even in individuals where the serum sample was positive, "in any case the detected amounts of free resveratrol were always very low (few ng/ml or less), in many cases below the limit of quantification...." *Id.* These data therefore "clearly demonstrated that resveratrol absorption after wine consumption is highly variable" and where any form of resveratrol could be detected, *trans*-resveratrol was detectable in very low amounts in only four of the subjects. (Vitaglione at 502-503, table 4). These data indicate that after consumption of substantial amounts of red wine, *trans*-resveratrol is not necessarily bioavailable, is rapidly metabolized, and where detectable, likely would not be present at significant levels. In particular, the low ng/ml levels of trans-resveratrol that Vitaglione observed in only 4 of the 25 subjects are approximately 1000 fold lower than the 5 to 30 μM (~1.2 – 6.9 μg/ml) concentrations used in the *in vitro* experiments reported in McNary and in Manna, (McNary at 3477; Manna, for example, at 6511-6512, fig. 2).

145. Walle et al. 2004 attached as **Exhibit 35** examined the absorption, bioavailability and metabolism of 14C-*trans*-resveratrol after oral (25mg) and i.v. (1.5mg) doses in six human volunteers. (Walle at 1378). While in its pure chemical form, *trans*-resveratrol demonstrated high oral absorption in this study, it also demonstrated "rapid and extensive metabolism, as determined by LC/MS, resulting in only trace amounts of unchanged resveratrol in the systemic circulation." (Walle at 1377). As Walle explains, "the most important question in this study was whether unmetabolized resveratrol could be detected in plasma...However, after all attempts to find measurable levels of resveratrol in plasma after the oral dose at any time point in the six

volunteers failed." (Walle at 1380). Indeed, even though this study administered resveratrol in amounts that Manna alleges would be present in a bottle of red wine, "the oral bioavailability of unchanged resveratrol established from our plasma data were close to zero." (Walle at 1381).

146. Finally, other *in vitro* studies not cited in the Office Action question the Examiner's assumption that Holmes-McNary and Manna demonstrate that even in cultured cells, pure trans-resveratrol necessarily reduces NF-kB activity. In particular, these studies observed that resveratrol did not necessarily affect NF-kB binding activity. As an example, Pendurthi et al. 1999 attached as **Exhibit 36** observed that a two hour pre-treatment with resveratrol had no significant effect on binding of NF-kB in endothelial cells stimulated with IL-1β, TNF-α or with LPS, even though it appeared to inhibit transcription of the tissue factor gene. (Pendurthi 1999 at 419, 422, 424, Figs. 4, 8 and 9). Such data demonstrate that the Examiner is incorrect in his assumption that resveratrol administration to cells necessarily reduces NF-kB activity, and provide further evidence that even if the Examiner were correct in assuming that trans-resveratrol would have been necessarily bioavailable after consuming red wine (which the above studies indicate is not accurate), consumption of red wine as described in the pre-April 21, 1989 art cannot anticipate any of the above claims.

IX. Protein Kinase C Inhibitors

A. Express Anticipation Rejection based on Meichle 1990 or Shirakawa 1989

147. I understand that the Examiner has rejected claims 1-6, 8, 9, 20-21, 25-27, 29, 31, 32, 36-40, 42-43, 47-51, 53-54, 58-62, 64-65, 69-73, 75-78, 80, 82, 84, 88-89, 93-97, 106-107, 109-110, 114-117, 192-193 and 197-201 as anticipated based on Meichle 1990 or Shirakawa 1989, improved copies of which are attached as **Exhibits 37** and **38**. I have reviewed each of these references and the claims. Each of these claims requires "reducing NF-kB activity." The

Examiner contends that each of these references teach administration of certain protein kinase inhibitors (staurosporine, H7 and H8) to reduce NF-kB activity in cells. (Office Action at 10-11). The Examiner further contends the references teach administration of H7 and H8 to reduce NF-kB activity so as to reduce NF-kB mediated gene transcription. *Id.* I respectfully disagree. One of skill in the art would not have understood any of these references to demonstrate that use of H7 or H8 reduced NF-kB activity. Moreover, as I discuss in more detail below, I disagree that any of these references describes or teaches any method of using H7 or H8 that would have carried out other additional elements required by the above claims.

(i) Express Anticipation Rejection based on Meichle 1990

148. Meichle reports various experiments conducted in two human leukemic cell lines, K562 and Jurkat cells. Meichle describes a series of experiments investigating whether in these cell lines, protein kinase C was necessary for the ability of tumor necrosis factor (TNF) to stimulate NF-kB binding activity. (Meichle at 8339, abstract). Meichle investigated this question by pretreating the cell with H7, H8 or staurosporine, three relatively non-specific protein kinase inhibitors. *Id.* Notably, the ability of TNF to stimulate NF-kB binding activity was unaffected by pretreatment with these inhibitors, and Meichle concludes that its ability to induce NF-kB binding activity did not involve activation of protein kinases, in particular protein kinase C (PK-C). *Id.* None of the experiments with TNF could therefore be relevant to any of the '516 patent claims. Meichle also purports to show that in contrast, pretreating the cell with either H7 or staurosporine reduced the ability of PMA to stimulate NF-kB binding activity. The Examiner contends that the use of H7 and staurosporine as described in Meichle would therefore anticipate '516 patent claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31, 32, 36-40, 53-54, 58-62, 64-65, 69-73,

75-76, 80, 82, 84, 88-89 and 93-97, if these claims were entitled only to a November 13, 1991 filing date. I respectfully disagree.

149. As discussed above, I understand all of the above claims as being directed to methods carried out on cells in which NF-kB activity has been induced. In this regard, the Examiner erroneously states that "Meichle teaches the reduction of NF-kB activity in induced cells..." (Office Action at 10). The experiments Meichle describes in which H7, H8 or staurosporine were administered to cells, including the specific experiments the Examiner relies on, (portions of Figs. 2 and 3) were all conducted by pretreating cells with these protein kinase inhibitors for 30 to 45 minutes before cells were stimulated with any substance. (Meichle at 8341-2, Figs. 2 and 3). As apparent from the discussion of these experiments, pretreatment was conducted to prevent a cellular response to inducing substances by inactivating PK-C, PK-A (and most likely other kinases) before stimulating the cell with any inducing agent. (See Meichle, at 8339, 8342). The use of these inhibitors as described by Meichle, therefore, was not in induced cells as assumed by the Examiner. None of these experiments would therefore have carried out the various methods described by the above claims.

150. The examiner also contends that Meichle teaches use of PK inhibitors to reduce NF-kB-mediated gene transcription by reducing NF-kB activity. (Office Action at 11). Additionally, the Examiner contends that because Meichle used a genetic construct comprising HIV LTR, Meichle either would anticipate or render obvious claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201, if these claims were entitled only to a November 13, 1991 filing date. I respectfully disagree. None of the experiments support these assertions. Meichle neither suggests nor provides any data showing that PK inhibitors inhibited gene expression induced by any agent, or could do so as a result of reducing NF-kB binding activity.

(ii) Express Anticipation Rejection based on Shirakawa 1989

152. Shirakawa et al. reports various experiments conducted in a mouse pre-B cell line 70Z/3, and in human natural killer-like cell line, YT. Shirakawa describes a series of experiments conducted to determine whether the ability of IL-1 in these cells to stimulate NF-kB binding activity required the activity of cAMP-activated kinases. (Shirakawa at 2424). Shirakawa examined this question using a methodology similar to Meichle, pretreating the cells with H8 for hours before stimulating cells with IL-1. (Shirakawa at 2426). The Examiner contends that the use of H8 as described in Shirakawa would have anticipated '516 patent claims 1-2, 5, 6, 8, 9, 20-21, 25-27, 29, 31, 32, 36-38, 40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89 and 93-97, if these claims were entitled only to a November 13, 1991 filing date. I respectfully disagree.

153. As discussed above, I understand all of the above claims as being directed to methods carried out on cells in which NF-kB activity has been induced. In this regard, the Examiner erroneously states that "Shirakawa teaches the reduction of NF-kB activity in induced cells using agents that inhibit protein kinase C" (Office Action at 12). The experiments Shirakawa describes in which H8 was administered to cells, including the specific experiments the Examiner relies on, (Fig. 1, portions of Fig. 2) were all conducted by pretreating cells with H8 for 2 hours before cells were stimulated with IL-1. (Shirakawa at 2426). Again, as in Meichle, pretreatment was performed to prevent a cellular response to IL-1 by inactivating PK-C and PK-A (and most likely other kinases) before stimulating the cell with IL-1. (Shirakawa at 2425-26). The use of these inhibitors as described by Shirakawa, therefore, was not in induced cells as assumed by the Examiner. None of these experiments would therefore have carried out the various methods described by the above claims.

154. The examiner also contends that based on CAT reporter assays described in Fig. 1, Shirakawa teaches use of PK inhibitors (H8) to reduce NF-kB-mediated gene transcription by reducing NF-kB activity, and therefore would have practiced the above claims. (Office Action at 12-13). I respectfully disagree. As discussed above, in this experiment cells were pretreated with H8 for two hours before being treated with IL-1. (Shirakawa at 2425). In fact, Fig. 1 indicates that before being treated with IL-1, the cells were washed and the H8 removed. The use of H8 in this experiment as described by Shirakawa, therefore, was not in induced cells and would not have reduced any induced effect in the cell, or practiced any of the claims, as the Examiner contends.

155. Moreover, one skilled in the art would be aware that each of the three PK inhibitors (H7, H8 and staurosporine) in particular at dose ranges used in Meichle and Shirakawa, are relatively unspecific and in addition to PK-C and PK-A affect numerous kinases. Significantly, by inhibiting other kinases, these agents can inhibit transcription unrelated to effects on PKC, or NF-kB. In particular, both H7 and H8 have been demonstrated to block gene expression and to inhibit mRNA chain elongation, most likely by inhibiting TFIIH kinase activity. (Yankulov et al. 1995; Kumahara et al. 1999) (**Exhibits 39 and 40**). Therefore, appropriate controls are a necessity in order to properly interpret the effects of such inhibitors on a transcriptional assay, such as CAT reporter assay, as being related to NF-kB. Shirakawa omits any such controls from CAT reporter experiment described in Fig. 1, for example, evidence that H8 would not affect expression of appropriately matched CAT construct lacking an NF-kB binding site. One skilled in the art would therefore not have read Shirakawa as teaching that one could use H8 to reduce In summary, therefore, for all the reasons above, one would not have read

Shirakawa to have taught reducing NF-kB-mediated gene transcription by reducing NF-kB activity, or to have carried out any of the above '516 patent claims.

X. 5-ASA

A. Inherent anticipation rejection based on Dew 1983

156. I understand that the Examiner has rejected claims 1-2, 5, 6, 8, 9, 20-27, 29, 31-38, 40, 53-62, 64-73, 75-80, 82, 84 and 88-97 as anticipated by Dew 1983 and explained by various cited non-prior art. (Office Action at 32). I have reviewed each of these references, the cited non-prior art and the above claims. The Examiner contends that each of these references teaches administration of 5-ASA for the treatment of ulcerative colitis. The Examiner contends further that cited non-prior art evidence shows that administration of 5-ASA as described in Dew would have necessarily reduced NF-kB activity "by a mechanism including phosphorylation [*sic*] of IkB" (Office Action at 33). The Examiner contends that therefore, each of these references inherently anticipates the above claims. I respectfully disagree.

157. Dew 1983 describes a clinical study conducted to compare use of a coated formulation of 5-amino salicylic acid to sulphasalazine in maintaining remission in patients who were in remission with ulcerative colitis. (Dew at 23). In fact, admission into the study "depended on normal sigmoidoscopic findings." *Id.* In other words, the study excluded any patient that was currently suffering from active ulcerative colitis disease. In this regard, the Examiner erroneously states that Dew "teaches administration of 5-ASA for the treatment of ulcerative colitis in humans." (Office Action at 33).

158. As noted above, I understand the above claims are all directed to methods carried out on cells in which NF-kB activity has first been induced by some inducing stimulus (external influence). Dew does not mention NF-kB, and does not describe any external influence that would have induced NF-kB activity. A representation of a eukaryotic cell in which NF-kB is not activated by an external influence is shown in Schematic 9 of **Exhibit 1**. The Examiner relies on data from Bantel, a non-prior art study conducted on patients with active ulcerative colitis, to assume that NF-kB activity would have been induced in the patients Dew studied. Such an assumption has no basis, as patients in Dew were not suffering from active ulcerative colitis, and represented an entirely different group of patients from those Bantel studied. In this regard, Bantel specifically notes that the patients studied were those "with an acute episode of moderative ulcerative colitis" as evidenced by endoscopic findings. (Bantel at 3453). Further, none of the other non-prior art references provide any evidence that NF-kB would be necessarily induced in patients in remission from ulcerative colitis. To the extent the Examiner relies on these other non-prior art references as evidence as to the effect of 5-ASA in induced cells, none of these other non-prior art references are relevant to use of 5-ASA in Dew. There is no evidence that use of 5-ASA described in Dew would have involved administration of 5-ASA to cells in which NF-kB activity was induced. Dew therefore could not have anticipated any of the '516 patent claims.

159. The Examiner also asserts that the patients in the Bantel study "were administered the same 5-ASA formulation (tradename MESALAZINE) in the same amount as in the Dew reference." (Office Action at 33). Such an assumption cannot be correct. Bantel specifically notes the data was generated from patients who were participating in a study "investigating the therapeutic effect of a *newly developed* mesalazine." (emphasis added) (Bantel at 3453). While

the 5-ASA Dew used was formulated by the investigators by coating 5-ASA with Eudragit S, the formulation used in Bantel consisted of pellets coated instead with Eudragit L. (Bantel at 3453). In the absence of any evidence demonstrating these formulations to be equivalent, one cannot assume they would necessarily act in the same manner.

XI. N-Acetyl -L-Cysteine

A. Express Anticipation Rejection based on Staal 1990

160. I understand that the Examiner has rejected claims 18 and 182-185 as anticipated based on Staal 1990. I have reviewed each of these references and the claims. All these claims require "reducing NF-kB activity." The Examiner contends that each of these references teach inhibiting TNF-α activation and signaling caused by TNF-α by reducing NF-kB activity by administration of N-acetyl-L-cysteine (NAC). One skilled in the art would not have understood any of these references to demonstrate that use of NAC reduced NF-kB activity.

161. Staal reports various experiments conducted in Jurkat cells, a human T cell lymphoma line, and 293.27.2, a cell line stably transfected with a CAT expression construct. As Staal notes, NAC is a drug which can prevent a reduction in intracellular thiol levels by serving as a precursor for the intercellular thiol, glutathione (GSH). (Staal at 9943). In previous studies, the authors had observed in certain T cell lines that NAC prevented TNF from stimulating certain cellular responses. (Staal at 9943). In this regard, Staal reported that TNF stimulation caused a rapid transient decrease in intracellular thiol levels. (Staal at 9944). Staal here reports a series of experiments conducted to examine whether the ability of NAC to prevent a TNF-

stimulated reduction in intracellular thiol levels also prevented TNF from activating NF-kB. (Staal at 9944).

162. As discussed above, I understand all of the above claims as being directed to methods carried out on cells in which NF-kB activity has been induced. In contrast, the aim of the Staal study was to determine whether NAC, by inhibiting thiol levels, could act on cells before they experienced stimulation. (Staal at 9944). In particular, in the experiments specifically relied upon by the Examiner, cells were always treated simultaneously with both NAC and TNF- α. (Staal at 9945-46). In these experiments, NAC therefore could not have inhibited TNF-α signaling by reducing induced NF-kB activity. None of these experiments have administered NAC to induced cells. Staal therefore could not teach the invention claimed in claims 18 and 182-185.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent.

Inder Verma, Ph.D.

Nov 9/06
Date

EXHIBIT C

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo

Docket No. 74753/JPW/GJG/PJS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

A Merged Proceeding of *Ex Parte* Reexamination Control Nos:

90/007,503 and 90/007,828
Filed April 4, 2005 Filed December 2, 2005

Merged Pursuant to May 4, 2006 Merger Decision
Group Art Unit: 3991 Examiner: B.M. Celsa

Patentees: David Baltimore, Ranjan Sen, Phillip A. Sharp, Harinder Singh, Louis Staudt, Jonathan H. Lebowitz, Albert S. Baldwin Jr., Roger G. Clerc, Lynn M. Corcoran, Patrick A. Baeuerle, Michael J. Lenardo, Chen-Ming Fan, and Thomas P. Maniatis

Patent No.: 6,410,516 B1 Serial No: 08/464,364

Issue Date: June 25, 2002 Filed: June 5, 1995

For : NUCLEAR FACTORS ASSOCIATED WITH TRANSCRIPTIONAL REGULATION

1185 Avenue of the Americas
New York, New York 10036

Mail Stop *Ex Parte* Reexamination
Central Reexamination Unit
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SECOND DECLARATION OF DR. INDER VERMA

I, Dr. Inder Verma, declare as follows:

1. I am the American Cancer Society Professor of Molecular Biology at The Salk Institute, Laboratory of Genetics, La Jolla, California. A copy of my *curriculum vitae* and a list of my publications are attached hereto as **Exhibit A**.

2. I have been retained by the patent owners' and licensee's counsel as a technical expert in this reexamination. I am being compensated at $600.00 per hour (or a maximum of $5,000 for a day). I was previous retained by the licensee's litigation counsel for purposes of the litigation in the District of Massachusetts, but I did not offer testimony in that litigation. I am not otherwise affiliated with the Patent Owners or the licensee.

3. I have previously presented my opinion in these proceedings in a Declaration dated November 9, 2006.

I. Scope of Opinion

4. I have been provided with, and asked to review, U.S. Patent No. 6,410,516, the claims as issued, the Final Office Action dated July 6, 2007, and the various references cited within that Office Action. I have been asked to provide an analysis of the scientific evidence relied on by the Examiner to reject certain claims of the '516 patent as expressly or inherently anticipated by these references. In particular, I have been asked to provide an analysis as to whether one of skill in the art would have understood these references to describe or disclose the elements of the '516 claims being rejected on the basis of these references. Where the rejection of certain claims has been made on grounds of inherency, I have also been asked to analyze whether there is any basis in fact and/or technical reasoning to support a determination that elements present in these claims would necessarily result from the teachings of the cited art. For the purpose of this declaration I have understood, that one skilled in this art in 1989 or 1991 would have at least a doctoral degree, e.g. a Ph.D. degree, in molecular biology or a related discipline, have at least 3 years of post-doctoral training, have knowledge in cell biology, biochemistry and genetics, and be well trained in laboratory methodologies.

5. The opinions set forth in this declaration are based on my professional knowledge and expertise, as indicated in my curriculum vitae, my review of the '516 patent and applications incorporated by reference therein, Orders Granting Requests for Reexamination 90/007,503 and Reexamination 90/007,828, and the Final Office Action dated July 6, 2007, including the documents cited in the Office Action, as well as additional documents cited in this declaration. For the purposes of this declaration I have been advised and understand that certain claims have been canceled, certain other claims have been confirmed patentable and the claims which will be pending and therefore under review are claims 6, 8-9, 14, 18, 64-80, 82, 84, 87-98, 139-148, 177-186.

II. Interpretation of the Claims

6. My interpretation of the claims is based on my understanding of how one of skill in the art would have understood the terms appearing in the claims in the context of the claims as a whole, in view of the description of the invention set forth in the patent.

7. All of the claims under review require the act of reducing induced NF-κB activity. For example, claim 6 recites "diminishing induced NF-κB-mediated intracellular signaling," claim 8 recites "modifying effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling," claim 9 recites "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling," claim 14 recites "a method for reducing bacterial lipopolysaccharide-induced expression of cytokines in mammalian cells, which method comprises reducing NF-κB activity in the cells so as to reduce bacterial lipopolysaccharide-induced expression of said cytokines," and claim 18 recites reducing "intracellular signaling caused by Interleukin-1 or Tumor Necrosis Factor-α." As such, all claims now under review

require that NF-κB activity be induced prior to the act of administering a compound that could reduce such induced NF-κB activity. Consequently, preventing or inhibiting activation of NF-κB does not fall within the scope of the claims now under review.

8. With respect to claim 8, I note that claim 8 recites a method for "modifying effects" of external influences such that the "effects" are "modified." The "effects" to which claim 8 refers are "NF-κB-mediated" and are therefore present only when NF-κB activity is present, i.e. induced NF-κB activity. We know that NF-κB is inactive if not induced by an inducing influence. Thus, in claim 8, the "effects" are present and there is induced NF-κB activity. Claim 8 would be meaningless if it was not understood that the "effects" are present because claim 8 required "modifying" the effects and requires the result to be "modified" effects. The "effects" and therefore NF-κB activity must be understood to be present in claim 8 because the terms "modifying" and "modified" cannot be referring to an effect that is not present.

9. Of the claims now under review, claims 71, 84, 95, 145, and 183 further require practicing the method on "human cells". I understand that a human cell is a cell that has not been transformed to contain bacterially or virally derived genetic elements. I also understand that a human cell infected with a virus by natural processes is still a human cell. Thus, reduction of NF-κB activity in cells which started as human cells but were transformed to contain bacterially or virally derived genetic elements does not fall into the scope of these claims.

10. Of the claims now under review, claims 64-69, 75-80, 88-93, 139-144, and 177-182 further require reducing induced NF-κB activity by interfering at a specific segment of the NF-κB pathway. As I explained in my November 6, 2006 Declaration, reduction of NF-κB activity can occur at multiple specific discrete segments in the NF-κB signaling pathway. Specifically,

NF-κB activity can be reduced "by decreasing the level of NF-κB not bound in an NF-κB:IκB complex" as recited in claims 64, 75, 88, 139, 177. NF-κB activity can also be reduced "by inhibiting the passage of NF-κB into the nucleus of cells" as recited in claims 65, 76, 89, 139 and 178; as well as by "reducing the binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB" as recited by claims 69, 89, 93, 144, 182; as well as "by inhibiting the modification of an IκB protein, which modification otherwise reduces IκB binding to NF-κB" as recited in claims 66, 77, 90, 144 and 179; as well as "by inhibiting degradation of an IκB protein" as recited by claims 67, 78, 91, 142 and 180; and as "by inhibiting the dissociation of NF-κB:IκB complexes" as recited by claims 68, 79, 92, 143 and 181. Thus, while all claims now under review require reducing induced NF-κB activity, certain further claims require doing so by interfering at a specific discrete segment of the NF-κB signaling pathway.

III. Cyclosporin A (CsA)

A. Express Anticipation Rejection Based on Schmidt 1990

11. I have read the Examiner's comments in the July 6, 2007 Final Office Action pertaining to the article by Schmidt et al. Journal of Virology (1990) 64(8):4037-4041 and I respectfully disagree on numerous points as summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 35-36 of July 6, 2007 Final Office Action)	
Schmidt teaches administration of Cyclosporin A (CsA) to cells which substantially reduced NF-κB activity in those cells	Schmidt et al. do not teach the administration of CsA to cells which reduced induced NF-κB activity. Since the CsA was always administered to cells at the same time as the agents that are purported to induce NF-κB activity, there was no induced NF-κB activity to be

thus inhibiting expression of genes whose transcription is regulated by NF-κB activity.	reduced. The CsA could, therefore, have only prevented or inhibited the activation of NF-κB by the simultaneously administered agents. Schmidt et al. provide no evidence that in their experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Schmidt et al.'s experiments showing prevention of activation of NF-κB activity (represented in Figure 1) are different from the experiments purporting to show inhibition of gene expression (represented in Figure 4). It is, therefore, incorrect for the Examiner to state that Schmidt et al. taught reduced NF-κB activity "thus" inhibited expression of genes. Moreover, Schmidt et al. do not disclose reducing the level of expression of "genes whose transcription is regulated by NF-κB activity." Schmidt et al.'s only experiment involving gene expression was conducted using a bacterial CAT gene (represented in Figure 4). A bacterial CAT gene is not regulated by NF-κB.
In addition, these references all utilize the HIV LTR promoter in their experiments and demonstrated that CsA reduced the expression of viral genes.	It is unclear to me what "references" the Examiner is referring to. However, Schmidt et al. do not use the HIV LTR promoter. Schmidt et al. use the κB enhancer from the HIV LTR, which is not the HIV LTR promoter (page 4037, bottom of first column). Schmidt et al. do not disclose use of any viral gene, much less demonstrate that CsA reduced the expression of viral genes. Schmidt et al.'s only experiment involving genes was conducted using a bacterial CAT gene under control of a viral κB enhancer (see legend Figure 4). A viral κB enhancer is not a viral gene.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1 or 2) or mammalian cells (e.g. claim 11) to	Claims 1-5 and 11 are no longer under review. The claims under review recite what they recite.

effect inhibited expression of a gene under transcriptional control of NF-κB. For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) to inhibit associated gene (viral gene such as HIV: (claims 1-4) expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling.
The Schmidt reference discloses that administration of Cyclosporin A (CsA) reduces NF-κB in cells (e.g. Jurkat cells) and therefore must inherently reduce NF-κB-regulated gene expression.	The CsA was always administered to cells at the same time as the agents that are purported to induce NF-κB activity, thus there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only inhibited the activation of NF-κB by the simultaneously administered agents. Schmidt et al. provide no evidence showing that in Schmidt et al.'s experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Schmidt et al.'s experiments showing prevention of activation of NF-κB (represented in Figure 1) are different from the experiments purporting to show inhibition of gene expression (represented in Figure 4). It is, therefore, incorrect for the Examiner to state that Schmidt et al. taught that administration of CsA "therefore must" reduce NF-κB-regulated gene expression. Moreover, Schmidt et al. do not disclose reducing the level of expression of "NF-κB-regulated gene expression." Schmidt et al.'s only experiment involving gene expression was conducted using a bacterial CAT gene (represented in Figure 4). A bacterial CAT gene is not regulated by NF-κB.
In particular, Schmidt utilized the Electrophoretic Mobility Shift Assay (EMSA) disclosed in the '516 patent to measure NF-κB activity to determine that "PHA-mediated induction of complexes	The full passage of Schmidt et al. being cited is "While PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA (Fig. 1, lane 6; no B or A shifts), the PMA-induced shift

binding to the kB enhancer was completely abrogated by [1ug/ml] CsA (Fig. 1, lane 6; no B or A shifts)...". See Schmidt at 4038. These results were confirmed using an NF-κB CAT reporter assay as described in the '516 patent, for example at Col. 17, line 66-Col. 18, line 23	was not at all affected (Fig. 1, lane 8). These data were confirmed with multiple different independent extracts. We conclude that very similar or identical κB complexes were activated via different pathways during stimulation with PHA versus PMA and that these pathways were distinguishable by their sensitivities to CsA." (page 4038). This clearly shows that Schmidt et al. was acutely aware that at least two pathways are involved in Schmidt et al.'s experiments and one was "not at all affected" by CsA.
Thus, Schmidt showed that Cyclosporin A reduced PHA-induced NF-κB activity and, therefore, reduced the expression of a gene (CAT) that was regulated by NF-κB.	Nowhere did Schmidt et al. show a reduction in PHA-induced NF-κB activity. Schmidt et al. show CsA prevents PHA-mediated activation of the CAT construct under control of the κB enhancer sequence of the HIV LTR (Figure 4, page 4039). Schmidt et al. provide no evidence that the CAT construct was regulated by NF-κB. Schmidt only shows that "CsA inhibited the PHA-derived activation signal but not the PMA signal." (page 4039, first column).
Accordingly, Schmidt described the use of Cyclosporin A at concentrations that reduce NF-κB activity and reduce NF-κB regulated gene expression.	Schmidt et al. do not show reduction of induced NF-κB activity in cells since NF-κB was not induced prior to CsA exposure in any experiment of Schmidt et al.
As such, and as shown in more detail in Exhibit G-1 of the 90/007,503 Request (herein incorporated by reference), the Schmidt references expressly anticipates at least claims 1-2, 5-9, 20-21, 25-29, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80-86, 88-89 and 93-97 of the '516 patent.	Schmidt et al. do not anticipate claims 6, 8 and 9, and claims dependent thereon because Schmidt et al. did not show reduction of induced NF-κB activity by any agent. Exhibit G-1 on page 13 confirms that "PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA" and that "CsA inhibited the PHA-derived activation." There is no evidence that CsA can reduce induced NF-κB activity.
Since Schmidt used the HIV LTR gene, Schmidt demonstrated that CsA reduced viral gene expression thereby anticipated	Schmidt et al. used the κB enhancer from HIV, not the HIV LTR. However, I

instant claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201.	understand that claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 are no longer under review.
Additionally, use of the HIV LTR gene by Schmidt renders instant claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 immediately envisaged, or alternatively, prima facie obvious in light of the fact that HIV LTR is responsible for regulating the expression of viral (HIV) genes. Therefore, it would have anticipated, or alternatively prima facie obvious, to regulate NF-κB activity as in Schmidt in order to affect associated viral (e.g. HIV) gene expression.	Schmidt et al. use only the κB enhancer from HIV, not the complete HIV LTR (page 4039, first column, also Figure 4). Neither the κB enhancer nor the HIV LTR constitute a gene.

Examiner Statement (Examiner Response- page(s) 36 of July 6, 2007 Final Office Action)	
Further, Schmidt teaches the addition of CsA during, as well as subsequent to, cell induced activation to reduce NF-κB activity. See Schmidt: "...addition of CsA during cellular activation phase completed abolished this binding" (page 4038 left column, last ¶) and "Direct addition of CsA to a prepared nuclear extract from activated cells had no effect on the factor binding..." (page 4038, right ¶, lines 1-8) (page 36)	The passage referred to has not been completely quoted in the July 6, 2007 Final Office Action. Schmidt et al. fully stated: "Direct addition of CsA to prepared nuclear extract from activated cells had no effect on the factor binding (data not shown), **which suggests that inhibition occurs during the activation of NFAT-1 or the κB complex**." (emphasis added, page 4038, second column) The full reading of the sentence rather than the partial fragment recited in the July 6, 2007 Final Office Action clearly states that 1) the effect of CsA occurs "during the activation" 2) the effect is on NFAT-1 or the κB complex, i.e. not on NF-κB, and 3) that addition of CsA to extract from cells that had previously been activated has no effect on factor binding meaning that transcription factors still bound after the addition of CsA.

Examiner Statement (Examiner Response- page(s) 40 of July 6, 2007 Final Office Action)	
Accordingly, Schmidt teaches (from two	Schmidt et al. do not describe any assay

different assays utilizing the HIV promoter) CsA inhibition of PHA and (PHA and PMA) NF-κB activated Jurkat cell binding and there is no evidence to rebut this teaching.	using the HIV promoter. It is unclear to me to what "Jurkat cell binding" refers. Schmidt et al. do not provide any explanation regarding what NF-κB activated Jurkat cell binding" may entail.

Examiner Statement (Examiner Response- page(s) 40 of July 6, 2007 Final Office Action)	
Thus the Schmidt reference teaches the ability of CsA to inhibit NF-κB activity in cells activated by PHA/PMA and thus enables the use of CsA to reduce intracellular NF-κB activity and inhibit NF-κB-mediated gene expression. (page 40)	Schmidt et al. do not teach the ability of CsA to inhibit NF-κB activity in cells activated by PHA/PMA since CsA was added at the same time as PHA/PMA, therefore in Schmidt et al., activation of NF-κB did not occur and there was no NF-κB activity to be reduced. Schmidt et al. provide no experimental evidence showing that NF-κB-mediated gene expression was reduced as a result of CsA reduced intracellular NF-κB activity. First, Schmidt et al.'s experiments showing prevention of activation of intracellular NF-κB (Figure 4) do not demonstrate a reduction in NF-κB activity because CsA was added at the same time as PHA/PMA, i.e. activation of NF-κB did not occur and therefore there was no NF-κB activity to be reduced. Further, Schmidt et al. do not use a gene whose expression is mediated by NF-κB since Schmidt et al. used a bacterial CAT gene. It is, therefore, incorrect for the Examiner to state that Schmidt et al. taught use of CsA "thus" inhibited intracellular NF-κB activity and NF-κB-mediated gene expression.

Examiner Statement (Examiner Response- page(s) 41 of July 6, 2007	

Final Office Action)	
Additionally it is art-recognized that CsA does affect NF-κB activity consistent with the Schmidt and Emmel teaching. The following three articles *rebut* patentee's assertion regarding NF-κB's lack of a role involving CsA.	Meyer et al. clearly teach, e.g. in the title of the reference, that "Cyclosporine A is an uncompetitive inhibitor of proteosome activity and **inhibits NF-κB activation**" (emphasis added) which does not mean that CsA reduces induced NF-κB activity. Inhibiting NF-κB activation is a method different from inhibiting induced NF-κB activity where NF-κB would have to be activated. Frantz et al. do not disclose any evidence wherein CsA was used. In fact, there is no mention of CsA in the Material and Method section indicating that this reagent was not used in Frantz et al. Roman-Blas et al. cite Meyer et al. and Frantz et al. to support the statement "Cyclosporin A inhibits the protease activity of the 20S proteasome complex preventing I-κBα degradation in murine macrophages, Jurkat lymphoma cells, and mouse and human T-lymphocytes" (page 843, first column). Thus Roman-Blas et al. do not disclose any experiment demonstrating CsA reduced induced NF-κB activity.

12. First, CsA cannot reduce induced NF-κB activity as required by the claims under review. All of the CsA references cited in the July 6, 2007 Final Office Action only show that CsA might prevent or inhibit activation of NF-κB. The cited references actually confirm that CsA cannot reduce induced NF-κB activity. As stated in Schmidt et al., "direct addition of CsA to a prepared nuclear extract from activated cells had no effect on the factor binding, including binding of κB-binding factors (data not shown), which suggests that inhibition occurs during the activation phase of NFAT-1 or the κB complex" (emphasis added, page 4038, second column, second

paragraph). Further, Kronke et al., which is of record, teach "when added 4 hr after induction, CsA did not alter TCGF mRNA levels" (emphasis added, page 5216, 2nd column, second paragraph). Induction of TCGF, also known as interleukin-2 (IL-2), is thought to be regulated by NF-κB. Finally, Reed et al., also of record, disclose "previous studies by us and by others have demonstrated that CsA (1 to 5ug/ml) does not interfere directly with IL 2 receptor function in that CsA fails to suppress IL 2-induced proliferation in long-term cultures of activated T cells" (emphasis added, page 153, 2nd column, first paragraph). Consequently, these results confirm that while CsA may possibly prevent induction of NF-κB activity, CsA cannot reduce induced NF-κB activity. Since, claims 6, 8 and 9, and claims dependent thereon, all require induced NF-κB activity, Schmidt et al. do not anticipate these claims. In fact, use of CsA cannot reduce induced NF-κB activity.

13. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has argued that Schmidt et al. teach administration of CsA to reduce NF-κB activity. I find no such teaching in Schmidt et al. Schmidt et al. only administer CsA to cells at the same time as the inducers PMA and PHA as described, e.g. in the Figure 1 legend on page 4038, to inhibit activation of NF-κB. Inhibiting activation is a method different from diminishing induced NFκB activity. As I have described in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot diminish induced NF-κB activity. Further, Exhibit G-1 relied on in the July 6, 2007 Final Office Action clearly states that "PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA" and "CsA inhibited the PHA-derived activation signal" thus reaffirming that CsA prevents activation of NF-κB but does not diminish induced NF-κB-mediated intracellular signaling as recited in claim 6.

14. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Schmidt et al. do not demonstrate that the administration of CsA modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. Schmidt et al. present data demonstrating that CsA can prevent stimulation of a cell by PHA to express a reporter gene construct comprised of a virally-derived enhancer sequence and a bacterially-derived chloramphenicol (CAT) resistance gene in a modified cell as depicted in Figure 4. Preventing stimulation is a method different from modifying effects of external influences that induce NF-κB activity. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot modify the effects of external influences that induce NF-κB-mediated intracellular signaling. Further, Exhibit G-1 relied on in the July 6, 2007 Final Office Action clearly states that "PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA" and "CsA inhibited the PHA-derived activation signal" (emphasis added) thus reaffirming that CsA prevents activation of NF-κB but does not modify effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

15. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Schmidt et al. do not provide any experiment demonstrating a CsA-mediated reduction in the level of expression of any gene which is activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Again, Schmidt et al.'s experiments involving the expression of the CAT reporter do not meet the requirements set forth in this claim.

16. Furthermore, CAT is not a gene endogenously regulated by NF-κB. CAT is a bacterial gene. Schmidt et al.'s experiment does not demonstrate that CsA reduced the level of expression of an activated gene because CsA was administered concurrently with PMA/PHA (Figure 4). Preventing activation is a method different from reducing the level of expression of genes which are activated. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot reduce the level of expression of genes activated by influences which induce NF-κB. Therefore, Schmidt et al. do not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

17. Claims 64-69 further limit claim 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by reciting reducing induced NF-κB activity by interfering at a specific segment of the NF-κB pathway. Schmidt et al. state that "CsA appears to inhibit step(s) in cellular activation which follows PHA or antigen receptor stimulation but does not inhibit the PMA signal" (emphasis added, page 4040, first column, second full paragraph). Nothing further about how CsA may impact NF-κB activity is described by Schmidt et al. I note that Exhibit G-1, relied on in the July 6, 2007 Final Office Action, has the following identical statement next to each of claims 64-69, 75-80 and 88-93: "Schmidt examined "nuclear extract," in an EMSA assay, p. 4037, and determined that "PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA (Fig. 1, lane 6; no B or A shifts)..." p.4038." Initially, this statement confirms that CsA "abrogated" the "induction" of complexes binding to the κB enhancer, which is a method different from reducing induced NF-κB activity which each of claims 64-67, 75-80 and 88-93 require. I also note that according to Schmidt et al., CsA treatment of "nuclear extracts" obtained from "activated cells" "had no effect on the nuclear

factor binding, including binding of κB-binding factors (data not shown), which suggests that inhibition occurs during the activation phase of NFAT-1 or the κB complex" (page 4038, second column). Furthermore, the statement in Exhibit G-1 relied on in the July 6, 2007 Final Office Action does not explain by which mechanism CsA operates, but rather describes only the observed result. Claims 64-69, 75-80 and 88-93, however, specifically require reduction of induced NF-κB activity by a specific mechanism which is not described in Schmidt et al.

18. I have reviewed the Schmidt et al. reference carefully and determined that the description of the experiments and methods presented does not enable one of skill in the art to reproduce Schmidt et al.'s work. The issue of experimental reproducibility is not a trivial one. In fact, a peer-reviewed journal entitled "Nature Protocols" has been introduced to address the issue of experimental reproducibility in scientific publications. As stated in a press release announcing the establishment of this journal, "the tips and shortcuts that researchers devise to improve a protocol rarely appear in print." See **Exhibit 1** hereto. Therefore, the lack of necessary protocol details in published reports is a serious problem that has warranted a high-profile journal to address it.

19. With respect to Schmidt et al., I would like to point out that the Schmidt et al. reference is a "Note" published in the Journal of Virology. Examination of the requirements for publication of a "Note" in this journal, a copy of which is attached as **Exhibit 2** hereto, show that "the Note format is intended for the presentation of brief observations that do not warrant full-length papers" and that "**Materials and methods should be described in the text, not in figure legends or table footnotes**" (page 11 of Journal of Virology, 2007 Instructions to Authors, bolding theirs, copy attached as **Exhibit 2**). Schmidt et al. do not fully articulate either in the text or in the figure legends, the methods used to prepare the reagents used in the study

presented, most notably, the method used to prepare the nuclear extracts derived from Jurkat cells. In the Figure 1 legend, Schmidt et al. state that "nuclear extracts (NE) were prepared from untreated (-) Jurkat cells or cells treated for 4.5 h with various agents...as described by Shapiro et al" (page 4038). Examination of the abstract for Shapiro et al., a copy of which is attached as **Exhibit 3** hereto, shows that Shapiro et al. used HeLa cells to generate nuclear extracts (see page 48, first column), a different cell line from the Jurkat cell line used by Schmidt et al. Further, Schmidt et al. make no reference of experimental conditions that would have been adjusted to accommodate the use of Jurkat cells, such as culture conditions, which makes replicating the experiment, using the exact conditions as Schmidt et al., impossible. In addition, the source of critical reagents, including CsA, PHA and PMA, as well as the methods used to prepare these reagents is not indicated. Considering there are many ways to generate these preparations, one can not be certain how Schmidt et al. generated reagents and could not reproduce the experiments in Schmidt et al. Further, on page 4039, first column, Schmidt et al. indicated that a modified protocol from DEAE using a hypotonic medium to swell the Jurkat cells before transfection. However, Schmidt et al. do not provide a protocol describing how this procedure was carried out. Schmidt et al. cite Lieber et al., a copy of which is attached as **Exhibit 4,** which do describe one method for modifying conditions for the transfection of a variety of cells (see Lieber et al., Table 1). However, the cell line used in the Schmidt et al. study is not included in the Lieber et al. study. In the Methods section of Lieber et al., multiple options are given as to how to carry out the osmotic transfection, however it is not clear which protocol Schmidt et al. followed for the transfection of the Jurkat cells used and therefore, one of skill in the art would be unable to reproduce this transfection experiment.

20. Also, rather than providing an explanation of the methods used to carry out the electrophoretic mobility shift assay, Schmidt et al. cited Staudt et al., a copy of which is attached as **Exhibit 5**, who also fail to provide instructions on how to perform these assays, instead citing Dignam et al., a copy of which is attached as **Exhibit 6**. Dignam et al. utilized HeLa cells for the preparation of nuclear extracts and stated on page 1476 in reference to assay conditions that "conditions were varied for other templates and in various experiments as indicated." Dignam et al. does not use the same cell line as Schmidt et al. and does provide the experimental details for Schmidt et al.'s work.

21. Moreover, Schmidt et al. fail to demonstrate any identification of the protein(s) involved in binding to the NF-κB oligonucleotides as determined by the EMSA assays presented in Figure 1. Without additional immunoprecipitation assays, western blotting with NF-κB antibodies or co-immunprecipitation assays that are routinely used for protein identification, one of skill in the art cannot reasonably conclude that NF-κB is the protein binding to the κB enhancer used in the EMSA assays. Consequently, the information provided by Schmidt et al. relating to experimental protocols and the lack of experiments that could confirm that NF-κB is the protein responsible for the mobility shift seen in Figure 1 may not be sufficient to enable someone of skill in the art to reproduce or reasonably interpret the experiments described. For this and previously discussed reasons, I believe that one skilled in the art could not readily reproduce Schmidt et al.'s work.

B. Express Anticipation Rejection Based on Emmel 1989

22. I have read the Examiner's comments in the July 6, 2007 Final Office Action pertaining to the article by Emmel et al. Science (1989) 246:1617-1620 and I respectfully disagree on numerous points as summarized in the table below.

Examiner Statement (Rejection Summary- page(s) 42-43 of July 6, 2007 Final Office Action)	
Emmel teaches administration of Cyclosporin A (CsA) to cells which substantially reduced NF-κB activity in those cells thus inhibiting expression of genes whose transcription is regulated by NF-κB activity.	Emmel et al. do not teach the administration of CsA to cells which reduced induced NF-κB activity. Since the CsA was always administered to cells at the same time as the agents that are purported to induce NF-κB activity, there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only prevented or inhibited the activation of NF-κB by the simultaneously administered agents. Emmel et al. provide no evidence showing that in Emmel et al.'s experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Emmel et al.'s experiments showing prevention of activation of NF-κB (represented in Figure 3) are different from the experiments purporting to show inhibition of gene expression (represented in Figures 1 and 2). It is, therefore, incorrect for the Examiner to state that Emmel et al. taught reduced NF-κB activity "thus" inhibited expression of genes. Moreover, Emmel et al. do not disclose reducing the level of expression of "genes whose transcription is regulated by NF-κB activity." Emmel et al.'s only experiments involving gene expression were conducted using a bacterial CAT gene (as represented in Figure 1 and 2). A bacterial CAT gene is not regulated by NF-κB.
In addition, these references all utilized the HIV LTR promoter in their experiments	It is unclear to me what "references" the Examiner is referring to. However, Emmel et al. do not use the HIV LTR in the experiments. Emmel et al. utilized the NF-κB site from the HIV LTR which is not the HIV LTR promoter (see legend of Figure 2).

and demonstrated that CsA reduced the expression of viral genes.	Emmel et al. do not disclose use of any viral gene, much less demonstrate that CsA reduced the expression of viral genes. Emmel et al.'s only experiments involving genes were conducted using a bacterial CAT gene under control of a viral NF-κB enhancer (Figure 2, legend). A viral κB enhancer is not a viral gene.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1 or 2) or mammalian cells (e.g. claim 11) to effect inhibited expression of a gene under transcriptional control of NF-κB. For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) to inhibit associated gene (viral gene such as HIV: claims 1-4) expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims 1-5 and 11 are no longer under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling
Similar to the Schmidt reference discussed above, the Emmel reference discloses that administration of Cyclosporin A (CsA) reduces NF-κB in cells (e.g. eukaryotic Jurkat cells which are a human leukemic T-cell line) that inherently reduces NF-κB regulated gene expression.	Emmel et al. do not teach that CsA reduced the amount of NF-κB in cells. In Emmel et al., the CsA was <u>always</u> administered to cells <u>at the same time</u> as the agents that are purported to induce NF-κB activity, thus there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only inhibited the activation of NF-κB by the simultaneously administered agents. Further, I do not understand a Jurkat cell line that has been transformed to express bacterially derived elements to be a "human cell." Emmel et al. provide no evidence showing that in Emmel et al.'s experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Emmel et al.'s experiments showing prevention of activation of NF-κB (represented in Figure 3) are different from the experiments purporting to show inhibition of gene expression (represented in Figures 1 and 2). It is, therefore, incorrect for the Examiner to state that

	Emmel et al. taught that the administration of CsA "therefore must" reduce NF-κB-regulated gene expression. Moreover, Emmel et al. do not disclose reducing the level of "NF-κB-regulated gene expression." Emmel et al.'s only experiments involving gene expression were conducted using a bacterial CAT gene (represented in Figures 1 and 2). A bacterial CAT gene is not regulated by NF-κB.
Like Schmidt, Emmel described the effect of CsA on Jurkat cells that were induced with PHA and PMA and CsA was shown (Fig. 3, .01-1ug/ml) to reduce NF-κB binding activity.	Emmel et al. do not teach the administration of CsA to cells which reduced induced NF-κB activity. Since the CsA was always administered to cells at the same time as the agents that are purported to induce NF-κB activity, there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only prevented or inhibited the activation of NF-κB by the simultaneously administered agents. Emmel et al. provide no evidence showing that CsA reduced induced NF-κB activity. Emmel et al.'s experiments purporting to show reduction of NF-κB activity (represented in Figure 3C) only demonstrates that exposure of the Jurkat cells to CsA in the presence of PMA and PHA prevented activation of NF-κB thereby resulting in the lack of NF-κB binding observed in Figure 3C.
In the CAT reporter assay, cells were transfected with a CAT reporter gene that was engineered to be regulated by HIV LTR gene, i.e., the gene had an NF-κB binding site incorporated into its regulatory region.	Emmel et al. use the NF-κB sequence from the HIV LTR, not the HIV LTR itself.
As shown in Fig. 2D, CsA significantly reduced NF-κB activity thereby reducing the NF-κB mediated expression of CAT.	Emmel et al. do not teach the administration of CsA to cells which reduced induced NF-κB activity. Since the CsA was always administered to cells at the same time as the agents that are purported to induce NF-κB activity, there

	was no induced NF-κB activity to be reduced. The CsA could, therefore, have only prevented or inhibited the activation of NF-κB by the simultaneously administered agents.
Additionally, as shown in Figure 3, 0.01-1ug/ml (10-10000 ng/ml) CsA was found to reduce NF-κB binding activity. Thus, Emmel described the use of CsA at concentrations that reduce NF-κB activity and reduce NF-κB regulated gene expression, and as such, and as shown in more detail in Exhibit G-1 of the 90/007,503 Request (incorporated by reference), the Emmel reference expressly anticipates at least claims 1-2, 5-9, 20-21, 25-29, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80-86, 88-89, and 93-97 of the :516 patent.	Emmel et al. do not teach the administration of CsA to cells which reduced induced NF-κB activity. Since the CsA was always administered to cells at the same time as the agents that are purported to induce NF-κB activity, there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only prevented or inhibited the activation of NF-κB by the simultaneously administered agents. Further, I understand claims 1-2, 5, 7, 20-21, 25-29, 36-40, 53-54, 58-62 and 81 are no longer under review. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling
Since Emmel used the HIV LTR gene, Emmel demonstrated that Cyclosporin A reduced viral gene expression thereby anticipating claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201.	Emmel et al. use the NF-κB sequence from the HIV LTR, not the HIV LTR itself. I understand that claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 are no longer under review. Moreover, Emmel et al. do not demonstrate a reduction of viral gene expression mediated by CsA. Emmel et al. use only the κB sequence from the HIV LTR linked to the bacterial CAT gene which does not comprise a gene regulated by NF-κB (see Figure 2, legend).
Additionally, Emmel's use of the HIV LTR gene renders instant claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 immediately envisaged, or alternatively, prima facie obvious since HIV LTR is responsible for regulating the expression of viral (HIV) genes.	Claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 are no longer under review.

Examiner Statement (Examiner Response-page(s) 44 of July 6, 2007 Final Office Action)	
Further, the Emmel reference teaches the ability to measure CsA inhibition of "stimulated" untransfected as well as transfected Jurkat cells upon incubation of these cells for 40 hours with PHA (2ug), PMA (50ng.ml) and CsA (concentrations of 10-1000 ng/ml).	Emmel et al. do not teach the use of "stimulated" untransfected cells. Emmel et al. provide no evidence of an untransfected cells treated first with PMA/PHA and then treated with CsA. Further, Emmel et al. do not describe the use of an untransfected cell under any conditions. The only two experiments using transfected cells describe the expression of specific constructs comprised of either the IL-2 enhancer (as described in the legend of Figures 1 and 2) or the NF-AT binding site, the AP-1 site or the NF-κB site from the HIV-LTR linked to the bacterially-derived CAT gene (see legend of Figure 2). Additionally, cells were never incubated for 40 hours with PHA/PMA and CsA. The legend of Figure 2 clearly states that "After a 40 hours incubation, cells were stimulated for 8 hours with PHA (2ug/mL), PMA (50ng/,L) with or without CsA (10ng/ml)" (page 1618).

23. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Emmel et al. teach administration of CsA to reduce NF-κB activity. I find no such teaching in Emmel et al. Emmel et al. only administer CsA to cells at the same time as the inducers PMA and PHA as described, e.g. in the legend of Figure 1: "cells were stimulated for 8 hours with PHA (2μg/ml) and PMA (50ng/ml) with and without CsA (10ng/ml)." Inhibiting activation is a method different from diminishing induced NF-κB activity. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot diminish induced NF-κB activity.

24. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Emmel et al. do not demonstrate that the administration of CsA modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. Emmel et al. present data demonstrating that CsA can prevent stimulation of a cell by PHA and PMA to express a reporter gene construct comprised of a virally-derived enhancer sequence (Figure 2, page 1618), a eukaryotic enhancer sequence from IL-2 (Figures 1 and 2), NF-AT (Figure 2) or AP-1 (Figure 2) and a bacterially-derived chloramphenicol (CAT) resistance gene in a modified cell as depicted in Figures 1 and 2. Preventing stimulation is a method different from modifying effects of external influences that induce NF-κB activity. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot modify effects of external influences which induce NF-κB-mediated intracellular signaling as required by claim 8.

25. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Emmel et al. do not provide any experiment demonstrating a CsA-mediated reduction in the level of expression of any gene which is activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Again, Emmel et al.'s experiments involving expression of the CAT reporter in the experiments described in Figure 1 and 2 do not teach reducing the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling as recited by the claim.

26. Moreover, CAT is not a gene endogenously regulated by NF-κB. CAT is a bacterial gene. Emmel et al.'s experiments do not demonstrate that CsA reduced the level of expression of an activated gene because CsA was administered concurrently with PMA and PHA (Figure 1-3). Preventing activation is a different method from reducing the level of expression of genes which are activated. Further, Exhibit G-1 relied on in the July 6, 2007 Final Office Action clearly states "Fig. 2D, showing a reduction in NF-κB-regulated gene expression in a CAT reporter assay" (emphasis added) thus reaffirming that Emmel et al. did not teach CsA reduces the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot modify effects of inducing influences. Therefore, Emmel et al. does not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

27. Claims 71, 84 and 95 are drawn to the practice of the method on "human cells." Emmel et al. do not disclose the use of a "human cell." Emmel et al. use Jurkat T cells, a human T cell line, in all experiments. The experiments described in Figures 3 and 4 utilize nuclear extracts generated from Jurkat cells treated simultaneously with CsA and PHA/PMA. However, the Jurkat cell lines described in the experiments presented in Figures 1 and 2 have been manipulated to express, at high levels, an artificial construct comprising "a series of internal deletion mutations of the IL-2 enhancer" linked to "the chloramphenicol acetyltransferase (CAT) gene" (page 1618, first column) or plasmids containing "the following regulatory sequences directing transcription of the CAT gene: (A) IL-2 enhancer...;(B) NF-AT, three copies of the NF-AT binding site...linked to the gamma fibrinogen promoter..;(C) AP-1, three copies of the AP-1 site linked to the SV40 promoter...;(D) NF-κB, one copy of the NF-κB site from the HIV-LTR

linked to the thymidine kinase promoter.." (page1618, Figure 2 legend). I don't understand these cells, which express various enhancer sequences used to drive expression of a bacterially-derived reporter gene, to be considered a "human cell."

28. Claims 64-69 further limit claim 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by reciting reducing induced NF-κB activity by interfering at a specific segment of the NF-κB pathway. Emmel et al. state that "CsA appears to exert its effects by inhibiting the isomerase-dependent refolding of proteins" (page 1619, second column). Nothing further about how CsA may impact NF-κB activity is described by Emmel et al. I note that Exhibit G-1, as relied on in the July 6, 2007 Final Office Action, has the following identical statement next to each of claims 64-69, 75-80 and 88-93: "Emmel examined nuclear extracts, in an EMSA assay "NF-κB binding was reduced 10-20% in nuclear extracts of CsA-treated cells." P. 1618." The statement in Exhibit G-1 does not explain how CsA operates, but rather describes only the observed result. Claims 64-69, 75-80 and 88-93, however, specifically require reduction of induced NF-κB activity by interfering at a specific segment of the NF-κB pathway.

29. I have reviewed the Emmel et al. reference carefully and determined that the description of the experiments and methods presented does not enable one of skill in the art to successfully reproduce the work presented. As I discussed in paragraph 18 above, the issue of reproducibility is not a trivial issue. First, the methods used to construct the reporter gene fusion comprising the IL-2 enhancer sequence linked to the bacterial CAT gene (Figure 1) are not disclosed in Emmel et al. Further, Emmel et al. do not describe from where the IL-2 enhancer sequence was derived nor do Emmel et al. provide information regarding the species of IL-2 enhancer used. Even if one were able to reproduce the experiment described in Figure 1, the result would not clearly indicate that NF-κB binds to the IL-2 enhancer sequence, driving expression of the CAT gene. I

have carefully reviewed Figure 1 and it is clear that there is no difference between cells expressing wildtype IL-2 enhancer linked to the CAT gene and cells that express a mutant version of the reporter gene construct wherein the NF-κB site has been disrupted. I would not understand the results of that experiment to demonstrate that NF-κB has any role in driving transcription of the CAT gene through the IL-2 enhancer. Likewise, the legend of Figure 2 does not provide adequate reference or instruction as to how the Jurkat cells were transfected nor does it describe how the cells were co-transfected. Critical information is missing such as the concentration of DNA used to transfect the cells as well as whether the same protocol used in Figure 1 was used to evaluate the CAT activity described in Figure 2, thus rendering it impossible for one of skill in the art to reproduce the experiments described in Figure 2. Additionally, the legend of Figure 4 states that "splenic lymphocytes were stimulated as described above for 2 hours in the presence or absence of CsA" (page 1619). It is not clear to me where in the reference Emmel et al. is referring. Additionally, the culture conditions for splenic lymphocytes are not described. Consequently, the experimental conditions, as described in Emmel et al., would not enable one of skill in the art to successfully reproduce the results presented and therefore, Emmel et al. is not an enabling reference.

C. Express Anticipation Rejection Based on Brini 1990

30. I have read the Examiner's comments in the July 6, 2007 Final Office Action pertaining to the article by Brini et al. Eur. Cytokine Net. (1990) 1(3):131-139 and I respectfully disagree on numerous points as summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 47-48 of July 6, 2007 Final Office Action)	
Brini teaches administration of Cyclosporin A (CsA) to cells which substantially	Brini et al. do not teach the administration of CsA to cells which reduced induced NF-

reduced NF-κB activity in those cells	κB activity. Since the CsA was <u>always</u> administered to cells <u>30 minutes before</u> the agents that are purported to induce NF-κB activity (page 132, second column), there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only prevented or inhibited the activation of NF-κB by the subsequently administered agents.
thus inhibiting expression of genes whose transcription is regulated by NF-κB activity.	Brini et al. provide no evidence showing that in their experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Brini et al.'s experiment showing prevention of activation of NF-κB activity (represented in Figure 3 and 4) are different from the experiments purporting to show inhibition of gene expression (represented in Figure 1). It is, therefore, incorrect for the Examiner to state that Brini et al. taught reduced NF-κB activity "thus" inhibited expression of genes. Moreover, Brini et al. do not disclose reducing the level of expression of "genes whose transcription is regulated by NF-κB activity." Brini et al.'s only experiment involving gene expression was using T lymphocytes stimulated with PHA after a 30 minute incubation with CsA (Figure 1, legend) and therefore have only inhibited activation of NF-κB by the subsequently administered agent.
In addition, these references all utilized the HIV LTR promoter in their experiments and demonstrated that CsA reduced the expression of viral genes.	It is unclear to me what "references" the Examiner is referring to. However, Brini et al. do not use the HIV LTR promoter. Brini et al. use the κB-like site from the HIV LTR, which is not the HIV LTR promoter. On page 132, Brini et al. disclose the use of the "HIV LTR <u>enhancer element.</u>" Brini et al. do not disclose the use of any viral gene, much less demonstrate that CsA

	reduced expression of viral genes. Brini et al. only use the HIV LTR enhancer element (page 132, second column). An HIV LTR enhancer element is not a viral gene.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1 or 2) or mammalian cells (e.g. claim 11) to effect inhibited expression of a gene under transcriptional control of NF-κB. For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) to inhibit associated gene (viral gene such as HIV; claims 1-4) expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims 1-5 and 11 are no longer under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling
The Brini reference discloses that administration of Cyclosporin A (CsA) reduces NF-κB in cells (e.g. T-cells) that inherently reduces NF-κB regulated gene expression.	Brini et al. do not teach that CsA reduced the amount of NF-κB in cells. In Brini et al. the CsA was <u>always</u> administered to cells <u>30 minutes before</u> the agents that are purported to induce NF-κB activity (page 132, second column), thus there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only inhibited the activation of NF-κB by the subsequently administered agents. Brini et al. provide no evidence showing that in Brini et al.'s experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Brini et al.'s experiments showing prevention of activation of NF-κB (represented in Figures 3 and 4) are different from the experiments purporting to show inhibition of gene expression (represented in Figure 1). It is, therefore, incorrect for the Examiner to state that Brini et al. taught that reduced NF-κB in cells "inherently reduces" NF-κB regulated expression of genes. Moreover, Brini et al. do not disclose reducing the level of expression of "genes whose transcription is regulated by NF-κB activity." Brini et al.'s only experiment

	involving gene expression was using T lymphocytes stimulated with PHA after a 30 minute incubation with CsA (Figure 1, legend) and therefore have only prevented or inhibited activation of NF-κB by the subsequently administered agent.
Particularly, Brini disclosed the use of 1 ug/ml CsA in human PBM (perphieral blood T-lympocytes) that had been induced with PHA. Brini concluded that "CsA reduced the PHA-induced binding of transactivating factors from T-cells and κB-like sequences which are present in the IL-2R alpha gene and in the HIV-1 LTR gene (Figures 3 and 4). See Brini at page 137.	Brini et al. do not demonstrate the induction of human PBM. The human PBMs had been treated with CsA for thirty minutes prior to exposure to PHA (page 132, second column) which would prevent or inhibit activation of the cells. The Examiner mischaracterizes the conclusion that CsA "reduced PHA-induced binding" since CsA was administered prior to stimulation with PHA. Therefore PHA could not "induce" binding of transactivating factors from T-cells and κB-like sequences.
Additionally, Brini reported the effects of CsA on expression levels of IL-2 Receptor-alpha (Brini at page 131 Abstract) which is taught by the '516 patent to be regulated by PHA-induced NF-κB activity in T-cells. See '516 patent, col. 17, lines 21-24 ("NF-κB is induced in T-cells by a transactivator (tax) of HTLV-1 or by PMA/PHA treatment and thereby activated the IL-2 receptor alpha gene and possibly the IL-2 gene".)	Brini et al. report that "pretreatment with CsA at 1μg/ml before cell activation caused a significant inhibition of the IL-2Rα mRNA induction." (page 143, first column).
Thus, Brini described the use of CsA at concentrations that reduce NF-κB activity and reduce NF-κB regulated gene expression and as such, and as shown in more detail in Exhibit G-1 of the 90/007,503 Request (incorporated by reference), the Brini reference expressly anticipates at least claims 1-2, 5-6, 8-9, 20-21, 25-27, 29, 36-38, 40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 85, 88-89, and 93-97 of the '516 patent.	Brini et al. do not describe the use of CsA to reduce NF-κB-regulated gene expression because Brini et al. did not conduct any experiment wherein NF-κB activity was induced prior to administration of CsA and therefore could not anticipate any of the pending claims. Further, I understand claims 1-2, 5, 7, 20-21, 25-29, 36-40, 53-54, 58-62 and 81 are no longer under review. Claim 6 and claims dependent thereon require diminishing induced NF-κB mediated intracellular signaling. Moreover, Exhibit G-1, relied on in the July 6, 2007 Final Office Action, stated

	that "These results are in accordance with Reed et al., who found that CsA inhibits Tac induction in mitogen-activated PBMC..." thus reaffirming that CsA prevents activation of NF-κB.
Additionally, since Brini used the HIV LTR gene, Brini, demonstrated that Cyclosporin A reduced viral gene expression thereby anticipating claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201.	I understand that claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 are not under review. Moreover, Brini et al. use the HIV LTR enhancer element, not the HIV LTR (page 132, second column). I do not undertand the HIV LTR or the enhancer element to comprise a "gene."
Additionally, Brini's use of HIV LTR renders instant claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201, immediately envisaged, or alternatively, prima facie obvious since HIV LTR is responsible for regulating the expression of viral (HIV) genes.	I understand that claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 are not under review. Moreover, Brini et al. use the HIV LTR enhancer element, not the HIV LTR (page 132, second column). I do not undertand the HIV LTR or the enhancer element to comprise a "gene."

Examiner Statement (Examiner Response – page(s) 49 of July 6, 2007 Final Office Action)	
Further, the Brini reference clearly addresses the ability of CsA to inhibit gene expression in a eukaryotic cell that is induced by PHA to release NF-κB and express protein: "Here we have examined the *inhibitory effect of CsA* on the activation of the IL-2Ralpha gene expression in primary human T lymphocytes induced by PHA (emphasis provided)."	Brini et al. clearly state that they have "examined the inhibitory effect of CsA on the activation of the IL-2Ralpha gene..." (page 132, first column) Brini et al. acknowledge that CsA is not reducing induced NF-κB activity but rather is inhibiting activation. Further, on page 137, Brini et al. state "when CsA was added directly to activated T-calls we did not find any interference with the binding detected by gel mobility shift assay []." This statement clearly indicates that even when the T-cells were activated, exposure to CsA has no effect.

Examiner Statement (Examiner Response- page(s) 45 of July 6, 2007 Final Office Action)	

As discussed supra, it s clear from the Brini article that PHA (and PMA) activates NF-κB to induce gene expression.	Brini et al. do not disclose the use of PMA in any experiment presented.

31. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Brini et al. teach administration of CsA to reduce NF-κB activity. I find no such teaching in Brini et al. Brini et al. administer CsA to cells thirty minutes prior to the inducer PHA as described, e.g., at page 132, to inhibit activation of NF-κB. Inhibiting activation is a method different from diminishing induced NF-κB activity. As I have described in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot diminish induced NF-κB activity. Further, in Exhibit G-1 relied on in the July 6, 2007 Final Office Action, it is stated that "CsA reduced the PHA-induced binding of transacting factors from T-cells and κB-like sequences which are present in the IL-2Rα gene and the HIV-1 LTR (Figures 3 and 4)." P. 137." However, the cited statement from Brini et al. is misleading. Further into the discussion Brini et al. state "...when CsA was added directly to activated T-cells we did not find any interference with the binding detected by gel mobility shift assay [unpublished observations]. From these data we can rule out the possibility that CsA acts directly on the activated NF-κB protein(s) system." (emphasis added, see page 137, bottom of first column). This reaffirms that CsA prevents activation of NF-κB activity.

32. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Brini et al. do not demonstrate that the administration of CsA modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. Brini et

al. present data demonstrating that CsA can prevent stimulation of a cell by PHA to express IL-2αR as depicted in Figure 1. Preventing stimulation is a method different from modifying effects of external influences that induce NF-κB activity. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot modify effects of external influences which induce NF-κB-mediated intracellular signaling as required by claim 8.

33. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Brini et al. do not provide any experiment demonstrating a CsA-mediated reduction in the level of expression of any gene which is activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Brini et al. present data demonstrating that CsA can prevent stimulation of a cell by PHA to express IL-2αR as depicted in Figure 1. Preventing stimulation is a different method from modifying the effects of extracellular influences that induce NF-κB-mediated intracellular signaling.

34. As I have discussed in paragraph 12 above, Schmidt et al. and others have shown that CsA cannot modify effects of inducing influences. Preventing activation is a different method from reducing the level of expression of genes activated by influences which induce NF-κB. Therefore, Brini et al. do not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

35. Claims 64-69 further limit claim 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by reciting reducing induced NF-κB activity occurs by interfering at a specific segment of the NF-κB pathway. Brini et al. state that "CsA inhibits the activation of NF-κB-like

factor(s)" (emphasis added, page 137, first column, second full paragraph). Brini et al. further note that "the NF-κB system has been shown to be under the regulation of a cytoplasmic inhibitor designated IκB, which after cellular stimulation releases that NF-κB protein which can reach the nucleus and react with the κB sequence [39]. We do not know yet how CsA blocks this step of activation." (emphasis added, page 137, second column, middle of paragraph). Furthermore, the statement in Exhibit G-1, does not explain how CsA operates, but rather describes only the observed result. Claims 64-69, 75-80 and 88-93, however, specifically require reduction of induced NF-κB activity by interfering at a specific segment of the NF-κB pathway.

36. I have reviewed the experimental techniques and methods described in Brini et al. and have determined that the description provided is not sufficient to enable one skilled in the art to replicate the work presented. As I discussed in paragraph 18 above, the issue of reproducibility is not a trivial issue. First, certain critical reagents used throughout this study, namely CsA and PHA, though referenced as to the supplier, have no lot numbers associated with them which one would require to determine if the batch of CsA used by Brini et al. would be equivalent to a batch used by another at later date. Further, the company which supplied the CsA, Sandoz, is no longer in operation and therefore one could not replicate the experiment described by Brini et al. Still further, the protocol cited for the preparation of nuclear extracts, Osborn et al., a copy of which is attached as **Exhibit 7**, describes the use of cultured immortalized cell lines and does not provide a protocol for the use of "primary T-cells pretreated with CsA" (page 135, first column). Therefore, one skilled in the art would not have the necessary information to successfully carry out the experiments as described by Brini et al.

D. Inherent Anticipation Rejection Based on the PDR 1985, Griffith 1982 and Griffith 1984 as evidenced by Holschermann 1997.

37. I understand that Examiner has alleged that the 1985 PDR, Griffith et al. I (1982) and Griffith et al. II (1984) inherently anticipate claims 6, 64-73, 8, 75-80, 82, 84, 9, and 88-97. I understand the Examiner's position to be that the method being claimed in claims 6, 8 and 9 and claims dependent thereon is described in the 1985 PDR, Griffith et al. I, Griffith et al. II based on Holschermann et al. I respectfully disagree. I have reviewed the claims, the 1985 PDR, Griffith et al. I and II and Holschermann et al. and determined that none of these references disclose the method of the claims under review. In the sections which follow, I first present my observations of the 1985 PDR, Griffith et al. I and Griffith et al. II, and then present my observation of the non-prior art reference, Holschermann et al., which the July 6, 2007 Final Office Action purports explains these references.

1985 PDR

38. I have reviewed the Examiner's comments in the July 6, 2007 Final Office Action regarding the 1985 PDR and disagree on several points. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that the 1985 PDR teaches administration of CsA to reduce NF-κB activity. I find no such teaching in the 1985 PDR. The 1985 PDR provides dosage and administration instructions for the use of CsA. Further, the 1985 PDR describes CsA as "a potent immunosuppressive agent which in animals prolongs survival of allogenic transplants involving skin, heart, kidney, pancreas, bone marrow, small intestine and lung. Sandimmune [cyclosporine] has been demonstrated to suppress some humoral immunity and to a greater extent, cell-mediated reactions such as allograft rejection, delayed hypersensitivity, experimental allergic encephalomyelitis, Freund's adjuvant arthritis and graft vs. host disease in many animal species for a variety of organs." (page 1811, third column).

Importantly, the 1985 PDR discloses a specific protocol of administration: "the initial dose of Sandimmune (cyclosporine) Oral Solution should be given 4-12 hours prior to transplantation.." (emphasis added, page 1813, first column). Therefore, the 1985 PDR cannot teach a method for diminishing induced NF-κB-mediated intracellular signaling as recited by claim 6.

39. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Importantly, the 1985 PDR discloses a specific protocol of administration: "the initial dose of Sandimmune (cyclosporine) Oral Solution should be given 4-12 hours prior to transplantation.." (emphasis added, page 1813, first column). Therefore, the 1985 PDR cannot teach, regardless of what Holschermann et al. disclose, the administration of CsA modifies the effects of external influences which induce NF-κB-mediated intracellular signaling and therefore does not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

40. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Importantly, the 1985 PDR discloses a specific protocol of administration: "the initial dose of Sandimmune (cyclosporine) Oral Solution should be given 4-12 hours prior to transplantation.." (emphasis added, page 1813, first column). Therefore, the 1985 PDR cannot teach, regardless of what Holschermann et al. disclose, CsA-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Further, the 1985 PDR does not describe reduction in

the level of expression of any gene that could be regulated by NF-κB. Therefore, the 1985 PDR does not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling as recited in claim 9.

41. Claims 64-69 further limit claim 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by reciting reducing induced NF-κB activity by interfering at a specific segment in the NF-κB signaling pathway. The 1985 PDR does not disclose any mechanism by which CsA reduces induced NF-κB. In Exhibit H-1, relied on in the July 6, 2007 Final Office Action, I note the same statement listed next to claims 64-69, 75-80 and 88-93: "Inherent. See Holschermann." This statement acknowledges that the 1985 PDR does not disclose a mechanism by which induced NF-κB can be reduced and therefore the 1985 PDR does not inherently anticipate these claims.

Griffith et al. I

42. I have reviewed the Examiner's comments in the July 6, 2007 Final Office Action regarding Griffith et al. I and disagree on several points. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Griffith et al. I teach administration of CsA to reduce NF-κB activity in cardiac transplantation recipients. I find no such teaching in Griffith et al. I. Griffith et al. I teach the administration of cyclosporine "orally just before operation" (page 324, second column). Even if one assumes that surgery induces NF-κB, which I am not certain that it does, administration of CsA prior to surgery is analogous to pretreatment and therefore at best prevents activation of NF-κB as stated in paragraph 12 where

it has been discussed that CsA cannot reduce induced NF-κB. Therefore, Griffith et al. I cannot teach a method for diminishing induced NF-κB-mediated intracellular signaling as recited by claim 6.

43. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Griffith et al. I teach the administration of cyclosporine "orally just before operation" (page 324, second column). Therefore, Griffith et al. I cannot teach, regardless of what Holschermann et al. disclose, the administration of CsA modifies the effects of external influences which induce NF-κB-mediated intracellular signaling and therefore does not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

44. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Griffith et al. I teach the administration of cyclosporine "orally just before operation" (page 324, second column). Therefore, Griffith et al. I cannot teach, regardless of what Holschermann et al. disclose, CsA-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Further, Griffith et al. I do not describe reduction in the level of expression of any gene that could be regulated by NF-κB. Therefore, Griffith et al. I do not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling as recited in claim 9.

45. Claims 64-69 further limit claim 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by reciting reducing induced NF-κB activity by interfering at a specific segment in the NF-κB signaling pathway. Griffith et al. I do not disclose any mechanism by which CsA reduces induced NF-κB. In Exhibit H-1, relied on in the July 6, 2007 Final Office Action, I note the same statement listed next to claims 64-69, 75-80 and 88-93: "Inherent. See Holschermann." This statement acknowledges that Griffith et al. I do not disclose a mechanism by which induced NF-κB can be reduced and therefore Griffith et al. I do not inherently anticipate these claims.

Griffith et al. II

46. I have reviewed the Examiner's comments in the July 6, 2007 Final Office Action regarding Griffith et al. II and disagree on several points. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Griffith et al. II teach administration of CsA to reduce NF-κB activity in cardiac transplantation recipients. I find no such teaching in Griffith et al. II. Griffith et al. II teach the administration of cyclosporine "orally 1 to 4 hours preoperatively and continued orally, or by nasogastric tube, every 12 hours postoperatively" (page 952, second column). Even if one assumes that surgery induces NF-κB, which I am not certain that it does, administration of CsA prior to surgery is analogous to pretreatment and therefore at best prevents activation of NF-κB as stated in paragraph 12 where it has been discussed that CsA cannot reduce induced NF-κB. Therefore, Griffith et al. II cannot teach, regardless of what Holschermann et al. disclose, a method for diminishing induced NF-κB-mediated intracellular signaling as recited by claim 6.

47. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Griffith et al. II teach the administration of cyclosporine "orally 1 to 4 hours preoperatively and continued orally, or by nasogastric tube, every 12 hours postoperatively" (page 952, second column). Therefore, Griffith et al. II cannot teach, regardless of what Holschermann et al. disclose, the administration of CsA modifies the effects of external influences which induce NF-κB-mediated intracellular signaling and therefore does not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

48. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Griffith et al. II teach the administration of cyclosporine "orally 1 to 4 hours preoperatively and continued orally, or by nasogastric tube, every 12 hours postoperatively" (page 952, second column). Therefore, Griffith et al. II cannot teach, regardless of what Holschermann et al. disclose, CsA-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Further, Griffith et al. II does not describe any reduction in the level of expression of gene that could be regulated by NF-κB. Therefore, Griffith et al. II do not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling as recited in claim 9.

49. Claims 64-69 further limit claim 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by reciting reducing induced NF-κB activity by interfering at a specific point in the NF-

κB signaling pathway. Griffith et al. II do not disclose any mechanism by which CsA reduces induced NF-κB. In Exhibit H-1, relied on by the July 6, 2007 Final Office Action, I note the same statement listed next to claims 64-69, 75-80 and 88-93: "Inherent. See Holschermann." This statement acknowledges that Griffith et al. II do not disclose a mechanism by which induced NF-κB can be reduced and therefore Griffith et al. II do not inherently anticipate these claims.

50. Further the Examiner has stated on page 59 of the July 6, 2007 Final Office Action "the 1985 PDR teaches administering the NF-κB inhibitor cyclosporine both prior and subsequent to the transplant, thus rendering patentee's "first to induce" argument moot." I have discussed the 1985 PDR relative to claim 6 previously in paragraph 37. I note the Examiner makes this point. I have read the 1985 PDR and the 1985 PDR states "the initial dose of Sandimmune (cyclosporine) Oral Solution should be given 4-12 hours prior to transplantation....This daily dose if continued postoperatively for one to two weeks and then tapered..." (page 1813, first column). As one of skill in the art, I understand this to mean that CsA was administered as a pretreatment to provide immunosupression in patients undergoing allogenic transplants (as stated on page 1811, third column). I do not understand the 1985 PDR to teach administration of "the NF-κB inhibitor."

Holschermann et al.

51. The Examiner cited Holschermann et al. Circulation (1997) 96(12):4232-4238 to purportedly explain what occurred in each of Griffith et al. I, Griffith et al. II and upon use of CsA as taught in the 1985 PDR. However, I do not understand how Holschermann et al. can explain what necessarily occurred in any of the three prior art references cited by the Examiner.

There are critical points that differ between Holschermann et al. and the prior art references. In addition to the points of distinction I discussed in my first Declaration dated November 9, 2006, there is no evidence that NF-κB had been activated in patients prior to the administration of CsA. As I discussed in paragraph 38 above, even if surgery were to induce NF-κB, and I am not certain that it does, the administration of CsA prior to surgery as is taught in the prior art is analogous to pretreatment and therefore at best prevents activation of NF-κB, but does not reduce induced NF-κB activity as required by the claims under review.

52. Further the protocols differ greatly between the prior art references and Holschermann et al. Summarized in the table attached as **Exhibit 8**, the protocol used by Holschermann et al. deviates greatly from the protocols outlined by the 1985 PDR, Griffith et al. I and Griffith et al. II. Patients in the prior art studies did not receive the same drug cocktail as those in Holschermann et al. and therefore it is unclear what effect, if any CsA had on the patients. One skilled in the art would expect a cocktail of different active drugs, as described, to result in the different patient outcomes. It is unreasonable to conclude that the results observed using one cocktail of active drugs in Holschermann et al. could possibly explain what previously happened when a different cocktail of active drugs was used in the prior art.

53. Further, the timing of administration of the drug cocktails differs greatly between the prior art and Holschermann et al. as summarized in the table attached as **Exhibit 8**. Holschermann et al. do not begin CsA treatment until 3 to 4 days after surgery, a highly relevant departure from the studies described in the prior art. Therefore Holschermann et al. cannot be used to explain what occurred in the prior art. As discussed in paragraph 12 above, CsA cannot reduce induced NF-κB activity.

54. Moreover, as someone of skill in the art, I do not understand the experiments described in Holschermann et al. to demonstrate that the administration of CsA reduces induced NF-κB. Holschermann et al. discloses a protocol wherein "PBMCs and monocytes/macrophages were prepared from blood samples drawn from cardiac transplant recipients before and after daily CsA administration" (page 4234, first column). Holschermann et al. go on to disclose that "measurement of the corresponding CsA plasma concentration in each blood sample revealed an increase of CsA blood levels from 233 ng/mL...in the sample before daily CsA administration" (page 4234, second column) which indicates that CsA was always present in the blood.

55. Further, I have critically examined Figures 3 and 4 of Holschermann et al. which the Examiner has pointed to in alleged confirmation of the ability of CsA to reduce the levels of a protein, tissue factor (TF) which is purported to be regulated by NF-κB. I respectfully disagree with the interpretation of the results set forth in the July 6, 2007 Final Office Action. First, in Figure 3 (a copy of which is attached as **Exhibit 9**), the sample loaded into lane 2, which is derived from blood collected from patients prior to the daily CsA administration has no detectable level of mRNA. Further, it is only after a six hour incubation can one observe a faint TF mRNA band, as indicated in lane 3. Notably, the sample collected from a patient after CsA administration and incubated for 6 hours shows no reduction in band intensity as shown in lane 6. It is only when the sample is incubated with LPS for 6 hours, can a prominent band be observed in lane 4, indicating an increase in TF mRNA transcription. These results are consistent with previously discussed references showing that the administration of CsA prevented the induction of TF mRNA by LPS as is indicated by the faint band in lane 7. Therefore, Figure 3 of Holschermann et al. show that CsA cannot reduce existing TF

transcription, though it appears to prevent activation of TF, a result entirely consistent with the literature discussed in paragraph 12.

56. Further, I have compared these TF mRNA transcription results with those presented in Figure 4 and I disagree with the interpretation of the data as set forth in the July 6, 2007 Final Office Action. First, the samples depicted in the "prior to" panel cannot correlate with a sample "prepared from blood mononuclear cells freshly isolated from transplant recipients before....CsA administration" (Figure 4, legend). If this were correct, Figure 3, lane 2, would depict the presence of TF mRNA, but it does not. The lack of activated TF mRNA, which is purported to be regulated by NF-κB, in samples obtained from patients prior to CsA administration indicates there is no activated NF-κB. The only conclusion that could correlate the results in Figure 3 to Figure 4 is that the samples obtained prior to CsA administration were incubated for 6 hours in the presence of LPS to stimulate NF-κB activity. In fact, in the legend for Table 2, such a step is described: "Mononuclear cell were isolated from peripheral blood samples of heart transplant recipients before and 4 hours after CsA administration, respectively, and assayed for TF activity after 6 hours of incubation with LPS" (page 4235). I therefore conclude that the only interpretation that can reconcile intense NF-κB band observed in the "prior to" sample in Figure 4 with the results shown in Figure 3 is that the samples underwent the 6 hour incubation with LPS. Otherwise, the disconnect between the lack of TF transcription (Figure 3, lane 2) compared to intense NF-κB bands observed in the "prior to" samples in Figure 4 still exists. Since NF-κB has been shown to activate transcription of TF, the only reasonable explanation is the one provided above. Consequently, not only did Holschermann et al. not carry out the therapy protocols set forth in the prior art, but the data obtained by Holschermann et al. does not demonstrate that CsA reduced induced NF-κB activity.

Non-reproducibility of Experiments

57. I do not understand the prior art references, 1985 PDR, Griffith et al. I and Griffith et al. II to provide enough detail to enable of one skill in the art to repeat their studies and arrive at the same results. The 1985 PDR provides dosage and administration instructions for the use of cyclosporine A. I understand as one skilled in the art that if I practice the method described in the 1985 PDR, I will observe a number of non-responsive patients or patients who exhibit adverse reactions (see table, page 1812). Therefore, the inherent variability in patient response to CsA renders it impossible for one to repeat the studies described in the prior art and obtain the same results. The 1985 PDR notes that "several study centers have found blood monitoring of cyclosporine useful in patient management" (page 1813, second column) and Griffith et al. II emphasizes this point, noting "the principal message is the lack of correlation between the dose of cyclosporine and the whole-blood level. Monitoring of the blood level in necessary to ensure that the administered dose provides a significant level of circulating cyclosporine" (page 954, first column). Thus, the lack of availability of the patient populations used in prior studies as well as the inherent variability in patients' responses to CsA would not enable one to practice the 1985 PDR, Griffith et al. I and Griffith et al. II studies and arrive at the same result.

58. Further, I have read the Examiner's comments in the July 6, 2007 Final Office Action pertaining to the experiments described by Holschermann et al., Griffith et al. I and Griffith et al. II and I respectfully disagree on numerous points as summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 54-57 of July 6, 2007 Final Office Action)	
PDR (1985), Griffith I and Griffith II teach cyclosporine A (CsA) administration of cells, which is shown from the teaching of	Discussed above

Holschermann, inherently reduces NF-κB activity and thus would inhibit expression of genes whose transcription is regulated by NF-κB activity.	
The inhibition is done by reducing binding of NF-B to NF-B recognition sites, which also decreases the level of NF-κB not bound in a NF-κB-IκB complex, inhibiting the passage of NF-κB into the nucleus of cells, inhibiting modification of an IκB protein, and inhibiting degradation of an IκB protein.	I do not find any of the prior art references or Holschermann et al. to describe any of the referenced requirements of the NF-κB not that CsA interferes at any of such segments to reduce induced NF-κB activity.
The PDR 1985, Griffith I and Griffith II references all teach the in vivo administration of CsA to cardiac transplant patients.	Discussed above
PDR 1985 teaches that CsA should be administered before and after surgery for 1-2 weeks at a dose of about 15 mg/kg/d, followed by a decrease of 5% per week to a final level of 5-10 mg/kg/day.	Discussed above
When monitoring whole blood levels, a 24 hour trough value of 250-800 ng/ml CsA appeared to minimize side effects and rejection effects.	The full citation in the 1985 PDR recites "While no fixed relationships have yet been established, 24 hour trough values of 250-800ng/ml (whole blood, RIA) or 50-300ng/ml (plasma, RIA) appear to minimize side effects and rejection events." (page 1813). I understand this to mean that by 1985 the optimal concentration of CsA in whole blood and plasma was still unclear and that monitoring of patients needed to occur to determine optimal concentrations.
Griffith I reports the administration of 5-10 mg/kg/d of CsA (average 8 mg/kg/d); while Griffith II reports the administration of 2-30 mg/kg/d) to obtain a targeted blood level of CsA of about 1000ng/ml.	Griffith et al. I do not disclose monitoring blood levels of CsA to obtain a target level of 1000ng/ml. The necessity of monitoring blood levels of CsA was disclosed in Griffith et al. II. Further, Griffith et al. II acknowledged that "It has been noted that an individual recipient's requirements for the drug vary after transplantation and that frequent incremental and decremental adjustments in the oral dose are necessary to achieve target level." (page 954, second column).
Holschermann provides extrinsic evidence that the PDR 1985, Griffith I, and Griffith	Discussed above

II references inherently anticipate the subject claims.	
Holschermann essentially repeated the tests disclosed in the Griffith I and II references by administering 3.4 ± 0.3 mg/kg/day CsA to cardiac transplant patients, resulting in blood levels of 681 ± 176 ng/ml.	Holschermann et al. did not "essentially repeat" the tests disclosed in the Griffith et al. I and II references. As summarized in Exhibit 8 and in paragraphs 52-53, critical differences exist between what Holschermann et al. did and what is described in the 1985 PDR, Griffith et al. I and II.
PBM cells were isolated from the blood of the patients before and after CsA therapy, and nuclear extracts from the cells were prepared.	Holschermann et al. note that "Measurement of the corresponding CsA plasma concentration in each blood sample revealed an increase of CsA blood levels from 233 ng/ml...before daily CsA administration to 691 ng/ml...in samples after daily CsA administration..." (page 4234, second column). I understand the presence of CsA in the plasma prior to the administration of CsA to be equivalent to "pretreatment" and therefore the patients in the Holschermann et al. study, at the time of evaluation, were being pretreated with CsA.
Holschermann then conducted an EMSA assay using nuclear extracts. (see figure 4) which is the same assay format taught by the '516 patent for determining whether compounds (i) reduce NF-κB activity and (ii) reduce binding of NF-κB to NF-κB recognition sites. See '516 patent, Col. 18, 1.52 – Col. 20, 1.25	I do not understand Holschermann et al. to describe experiments demonstrating reducing NF-κB activity and reducing binding of NF-κB to NF-κB binding sites. As discussed above, Holschermann et al. demonstrate the inhibition of NF-κB activation.
Holschermann confirms that administering CsA to cardiac patients as taught by the prior art PDR 1985 and Griffith I and II references necessarily inherently reduces NF-κB activity (and binding of NF-κB to NF-κB recognition sites): In cells obtained from transplant recipients during low baseline CsA blood levels (before CsA administration), strong NF-κB binding activity was detected (Fig. 4), whereas cells separated from blood in the presence of high CsA concentrations exhibited decisively reduced NF-κB binding activity. Specifically of the binding reaction was shown by the competition with unlabeled	Figure 4 of Holschermann et al. cannot depict NF-κB levels in blood isolated from patients immediately prior to and following CsA administration. The levels of NF-κB depicted in Figure 4 do not comport with the TF mRNA levels depicted in Figure 3. In blood isolated from patients prior to their daily CsA dosage, there was no TF mRNA observed (see Fig. 3, lane 2). Only after a 6 hour incubation with LPS (Fig. 3, lane 4), could levels of TF mRNA be observed that would be consistent with the amount of NF-κB levels observed in Figure 4. Therefore, it is not accurate to state that Holschermann et al. depicts CsA reducing

consensus oligonucleotides. Id. At 4236	NF-κB activity. See also my discussion in paragraphs 51-56 above.
Holschermann also showed that the administration of CsA to these patients as taught in the prior art PDR 1985 and Griffith I and II references reduced Tissue Factor (TF) gene transcription, which is recognized as being regulated by NF-κB: "Indeed, the marked activation of the NF-κB transcription factor, which is known to play a major role in the regulation of the TF gene, was prevented in the presence of high CsA blood concentrations." Id at 4237	The data presented in Figure 3 of Holschermann et al. does not depict the reduction of TF mRNA after administration of CsA. A TF mRNA transcript is observed only after the sample isolated from patients has been incubated for 6 hours regardless of whether the sample was obtained prior to or after CsA administration (Fig. 3, lanes 3 and 4). The sample in Lane 2 does not show any TF mRNA. Further, the blood sample taken after CsA treatment and allowed to incubate for 6 hours show no difference from the sample obtained prior to CsA treatment and processed under the sample conditions (Fig. 3, lanes 3 and 6). Finally, only the samples obtained after CsA treatment and incubated with LPS show any difference, which difference is the reduction in TF mRNA due to CsA preventing activation of TF.
Thus, CsA, as administered in PDR 1985 and Griffith I and II: -inhibited expression of a gene whose transcription is regulated by NF-κB (instant claims 1 and 2 and their dependent claims); -diminished NF-κB-mediated intracellular signaling (clm 6 and dependent claims); and –reduced NF-κB mediated effects of external influences (claims 7 and 8 and dependent claims).	It is an inaccurate statement on multiple grounds as I explained above in paragraphs 51-56.
Since CsA was shown to reduce binding of NF-κB in an EMSA assay which measures binding of NF-κB to NF-κB recognition sites, Holschermann confirms that the prior art administration of CsA to cardiac patients reduces NF-κB activity by "reducing binding of NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB" (e.g. claims 25, 36 and 58).	Claims 25, 36 and 58 are not under review.
Additionally, because unbound NF-κB translocates to the nucleus, the reduced binding activity in the nucleus of cells reflected in Holschermann means that CsA, as administered in PDR 1985 and Griffith I	Claims 20, 21, 32, 53 and 54 are not under review.

and II, necessarily reduced NF-κB activity by: A. "decreasing the level of NF-κB not bound in any NF-κB-IκB complex" (e.g. claims 20, 31 and 53; and B. "inhibiting the passage of NF-κB into the nucleus of cells (e.g. claims 21, 32 and 54).	
Furthermore, as Holschermann indicates, CsA is recognized as being able to "abolish the inducible phosphorylation and degradation of the cytoplasmic inhibitor protein IκB." Id. At 4237 (citing Alkalay).	I understand Alkalay et al., the reference cited by Holschermann et al. to describe the pretreatment of Jurkat cells to prevent IκB modification. As Alkalay states "Cyclosporin A (CsA)...was used at a concentration of 400ng/ml for 30 min prior to cell stimulation." (page 1295, first column). Therefore, like all previous studies, cells were pretreated with CsA, thus preventing activation of NF-κB rather than reducing induced NF-κB as is required by the claims.
Holschermann confirms that this effect on degradation of IκB is the mechanism by which CsA reduced NF-κB in these cardiac patients.	Holschermann et al. provide no evidence that IκB is degraded and does not confirm that "degradation of IκB is the mechanism by which CsA reduced NF-κB activity." As such, the art shows CsA cannot reduce induced NF-κB as claimed by the claims under review.
Thus, Cyclosporin A when administered to humans as in the PDR 1985 and Griffith I and II references reduces NF-κB activity by: A. "inhibiting modification of an IκB protein, which modification otherwise reduces IκB binding to NF-κB" (e.g. claims 22 and 33); and B. "inhibiting degradation of an I-κB protein" (e.g. claims 23 and 34).	It is inaccurate to state "thus" cyclosporine when administered as in the PDR 1985 and Griffith et al. I and II references reduces NF-κB activity by any of the mechanisms described. Holschermann et al. cannot comport the results obtained in Figure 3 to those obtained in Figure 4 therefore I do not understand Holschermann et al. to support the idea that CsA has effect on NF-κB activity. Further, Holschermann et al. do not describe any experiment wherein the mechanism of inhibition is tested and therefore cannot explain what necessarily occurred in the prior art references.
Finally, as demonstrated by Holschermann, the PDR 1985 and Griffith I and II reference CsA administration to human patients reduced NF-κB activity in those patients' peripheral blood mononuclear cells (PBM's comprised of lymphocytes and monocytes) which anticipates: 1)	Claims 1, 2, 5, 26-27, 29, 37-38, 40, 61-62 are not under review.

eukaryotic cells (claims 1-2, 5 and 9); 2) mammalian cells (claims 26, 37, 70, 82 and 94); 3) human cells (claims 27, 38, 71, 84 and 95); 4) immune cells (claims 61 and 72); and 5) lymphocyte cells (claims 29, 40, 62, 73 and 97).	
It is noted that the dosage and blood levels of CsA shown by Holschermann to reduce NF-κB activity is slightly lower than the dosages and blood levels of CsA taught in the PDR 1985, Griffith I and II references.	Griffith et al. II note that "the average dose of 8 mg/kg/day [CsA] resulted in a blood level of 1,089 ng/ml" (page 953, first column). It is not clear to me whether the administration of 3.4 mg/kg/d of CsA as taught by Holschermann et al. would be sufficient to reach the critical 1000ng/ml blood serum concentration of CsA as suggested by Griffith et al. II in every patient.
Accordingly, an even greater reduction in NF-κB activity would result from the prior art administration of CsA to patients as described these references than shown in Holschermann.	There is no indication that "an even greater reduction in NF-κB activity would result from the prior art administration of CsA" for the reasons explained above.
Moreover, regardless whether the effect of CsA in reducing NF-κB activity and resulting monocyte TF activation is direct (by directly affecting monocytes) or indirect (by interfering with stimulatory lymphocytes), Holschermann shows that CsA, as administered in the prior art references reduces NF-κB activity and resulting TF gene expression.	Discussed above
Thus its administration of cardiac transplant patients as taught in PDR 1985, Griffith I and II anticipates at least claims 1-2, 6, 8-9, 20-27, 29, 31-38, 40, 64-73, 75-80, 82, 84, 86 and 88-97 of the '516 patent, as set forth in more detail in Exhibit H-1 of the 90/007,503 Request (incorporated by reference).	Claims 1-2, 20-27, 29, 31-38, 40, and 86 are not under review. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling

Examiner Statement (Examiner Response-page(s) 63 of July 6, 2007 Final Office Action)	
Upon viewing the original Holschermann document, even in black and white, the Examiner was able to visualize in Fig. 3 discernable bands representative of Lane 2	I disagree that Lane 2 in Figure 3 depicts any discernable TF mRNA. Lane 3 does not depict "more pure sample" as the Examiner has stated, but rather samples

(*ex vivo* unpurified) and particularly Lane 3 (more pure sample), both of which represent the presence of TF mRNA in the *ex-vivo* blood samples.	that have been incubated for 6 hours. It is only when the sample has been incubated for 6 hours in the presence of LPS that a strong TF mRNA signal can be observed.

Examiner Statement (Examiner Response-page(s) 63 of July 6, 2007 Final Office Action)	
Thus, contrary to patentee's argument, the presence of NF-κB in human transplant patient's PBM's shown in the EMSA assay Fug. 4 Lanes 1-3 correlates with the moderate TF mRNA expression in the same transplant patient as indicated in the Fig. 3 assay.	I respectfully disagree with the Examiner's conclusion that the results in Figure 3 comport with those in Figure 4 for the reasons stated above in paragraphs 53-54. Further, Holschermann et al. do not state that the "same transplant patient" was used for the experiments shown in Figures 3 and 4.

Examiner Statement (Rejection Summary –page(s) 66-68 of July 6, 2007 Final Office Action)	
Reed teaches cyclosporine A (CsA) administration of cells, which, as is shown in Brini, inherently reduces NF-κB activity and thus would inhibit expression of genes whose transcription is regulated by NF-κB activity.	Reed et al. do not teach the administration of CsA to cells inherently reduces NF-κB activity. Reed et al. administer CsA "20 to 30 min before other reagents" (page 150, second column). Thus there was no induced NF-κB activity to be reduced. The CsA could, therefore, have only inhibited the activation of NF-κB by the subsequently administered agents.
The inhibition is done by reducing binding of NF-κB to NF-κB recognition sites, which also decreases the level of NF-κB not bound in a NF-κB-IκB complex, inhibiting the passage of NF-κB into the nucleus of cells, inhibiting modification of an IκB protein, and inhibiting degradation of an IκB protein.	It is an inaccurate statement on multiple grounds as I explained above in paragraphs 51-56.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1, 2 or 5) or mammalian cells (e.g. claim 26) to effect inhibited expression of a gene under transcriptional control of NF-κB.	Claims 1, 2, 5 and 26 are not under review.
For example, NF-κB activity can be effected by diminishing induced NF-κB	Claims 5, 7 and 20 are not under review.

mediated intracellular signaling (claims 6-9) or decreasing the level of NF-κB not bound in an NF-κB-IκB complex (claim 20) to inhibit associated gene expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling
The in vivo effect of CsA in reducing NF-κB activity in human PBM cells is confirmed by tests using CsA in human PBM cell cultures.	I do not understand which reference the Examiner is referring to in this statement.
For example, Reed taught the prior art use of CsA in human PBM cell cultures that had been induced with phytohaemagutinin (PHA).	Reed et al. teach pre-treatment of PBMC with PHA-P 20 to 30 minutes prior to stimulation (page 150, second column).
In particular, Reed teaches that CsA (1ug/ml) significantly reduced PHA induced IL-2 R alpha gene transcription (Figs. 1 and 2) and Tac antigen surface expression (Fig. 3b) in human PBM cells.	Reed et al. do not teach that CsA significantly reduced PHA induced IL-2 R alpha gene transcription and Tac antigen surface expression. Reed et al. disclose that "CsA blocked the PHA-mediated induction of IL-2 responsivity in PBMC cultures.." (page 152). Reed et al. further state on page 153, "CsA fails to suppress IL-2 induced proliferation in long-term cultures of activated T-cells." Reed et al. also do not teach reduction of PHA-induced IL-2 R alpha gene transcription and Tac antigen surface expression since Reed et al. do not induce cell cultures prior to the administration of CsA.
Reed also found that CsA reduced IL-2 gene transcription: "1ug/ml CsA and 10^{-4} M DEX completely blocked the PHA-induced accumulation of mRNA for IL 2 (Fig. 2). Id at 151	Reed et al. do not demonstrate a reduction in IL-2 gene transcription. As Reed et al. state, "CsA...completely blocked the PHA-induced accumulation of mRNA for IL-2." (emphasis added, page 152, first column)
Several years later, Brini also looked at the effect of Cyclosporin A on IL-2 Receptor-α production and Tac antigen surface expression and confirmed Reed's results: These results are in accordance with Reed et al., who found that CsA inhibits Tac induction in mitogen-activated PBMC. The CsA-mediated reduction in Tac antigen expression of T-cells was reflected by a decrease in the steady state mRNA levels of the IL-2Rα chain (Figure 2). Brini at 137 (citations omitted).	Brini et al., like Reed et al., administered CsA 30 min prior to stimulation (see Brini et al., page 132, second column; see Reed et al., page 150, second column). Therefore, Brini et al. and Reed et al. both demonstrate preventing or inhibiting activation of NF-κB.
Brini then went on, however, to show that	Brini et al. do not disclose a reduction of

CsA, as administered in the prior art, reduced binding of NF-κB to NF-κB recognition sites for more than one NF-κB mediated gene: CsA reduced the PHA-induced binding of transacting factors from T- cells and κB-like sequences which are present in the IL-2Rα gene and in the HIV-1 LTR (Figures 3 and 4). Brini at 137.	NF-κB binding to recognition sites for more than one NF-κB mediated gene. Brini et al. do not demonstrate that binding occurred prior to administration of CsA since Brini et al. always incubated the cells with CsA at least 30 minutes prior to stimulation (see page 132, second column). Further, the use of κB-like sequences found in the IL-2Rα gene and in the HIV-1 LTR to not comprise a gene and therefore I do not understand these elements to be NF-κB-mediated genes.
Notably, Brini assessed NF-κB activity in an EMSA (Fig. 5) using the same HIV-1 LTR site as used by the instant '516 patent to assess NF-κB activity and binding.	Brini et al. use the "HIV-LTR enhancer element" (page 132, second column), not the HIV-1 LTR.
As such Brini concluded that CsA regulated IL-2Rα gene expression by reducing activation of NF-κB: taken together, these results suggest that one of the effects of CsA in the regulation of the IL-2Rα chain expression in human peripheral T lymphocytes is on the activation of sequence specific DNA-binding proteins which recognize sequences containing the NF-κB binding site. Brini at 137.	Brini et al. do not disclose reducing <u>induced</u> NF-κB activity. Brini et al. note that "when CsA was added directly to <u>activated</u> T-cell we did not find any interference with the binding detected by gel mobility shift assay.." (emphasis added, page 137, first column).
Using the same inducer, the same immune cells, and the same concentration of CsA as in Reed, Brini showed that CsA reduced NF-κB activity and NF-κB-medicated IL-2 Receptor-α gene expression in PBM cells.	Brini et al. do not show any reduction in NF-κB activity mediated by CsA since the cells were not induced prior to CsA administration.
In this respect, the instant '516 patent confirms that the IL-2 receptor alpha (IL-2Rα) gene is regulated by PHA-induced NF-κB activity in T-cells. Col. 17, lines 21-24 ("NF-κB is induced in T-cells by a trans-activator (tax) of HTLV-1 or by PMA/PHA treatment and thereby activates the IL-2 receptor alpha gene and possibly the IL-2 gene.").	Unlike the '516 patent, Brini et al. and Reed et al. either pre-treat cells with CsA prior to stimulation or administer CsA concurrently with the agent purported to induce NF-κB activity. The claims under review from the '516 patent, specifically claim 6 and claims dependent thereon require diminishing induced NF-κB activity.
Thus, Brini and the related human T-cell culture studies discussed below confirm that CsA necessarily and inherently reduced NF-κB activity and resulting gene	Brini et al. and the related cell culture studies do not teach that CsA necessarily and inherently reduced NF-κB activity and resulting gene expression in PBM cell

expression in PBM cell cultures as taught in Reed, thus anticipating at least claims 1-2, 5-6, 8-9, 20-21, 25-27, 29, 31-32, 36-38, 40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89, and 93-97.	cultures as taught by Reed et al. since none of these references can demonstrate that the cells were induced prior to CsA administration. The cells were always either pretreated with CsA prior to addition of the stimulus purported in induce NF-κB or treated with CsA and the purported stimulus at the same time.

Examiner Statement (Rejection Summary-page(s) 74-75 of July 6, 2007 Final Office Action)	
Kronke, or Siebenlist teach cyclosporine A (CsA) administration of cells, which, as is shown from the teachings of Schmidt and Emmel, inherently reduces NF-κB activity.	Kronke et al., Siebenlist et al., Schmidt et al. and Emmel et al. all fail to demonstrate that the cells in their respective experiments were induced prior to CsA treatment and therefore cannot teach that CsA administration of cells inherently reduces induced NF-κB activity.
The inhibition is done by reducing binding of NF-κB to NF-κB recognition sites, which also decrease the level of NF-κB not bound in a NF-κB-IκB complex, inhibiting the passage of NF-κB into the nucleus of cells, inhibiting modification of an IκB protein, and inhibiting degradation of an IκB protein.	I do not find any of the prior art references or Kronke et al. or Siebenlist et al. to describe any of the referenced segments of the NF-κB pathway nor that CsA interferes at any of such segments to reduce induced NF-κB activity.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1, 2 or 5) or mammalian cells (e.g. claim 26) to effect inhibited expression of a gene under transcriptional control of NF-κB.	Claims 1, 2, 5 and 26 are not under review.
For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) or decreasing the level of NF-κB not bound in an NF-κB – IκB complex (claim 20) to inhibit associated gene expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims 5 and 20 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling
The effect of CsA on reduction of NF-κB activity as administered in the prior art is also confirmed by tests involving the use of CsA in human T-cell (Jurkat) cultures including two prior art references, Kronke and Siebenlist.	Kronke et al. disclose "cloned leukemic T cells....induced with PHA..and PMA...for 6 hr in the presence or absence of CsA" (page 5215, second column). Siebenlist et al. teach "...a 2.5- and 4.5-h stimulation were done also in the presence of CsA.."

	(Figure 2, legend). Therefore, neither Kronke et al. nor Siebenlist et al. disclose reducing induced NF-κB activity.
Using the same inducers, the same human immune cells, and the same concentration of CsA taught in Kronke and Siebenlist, Schmidt and Emmel provide confirming extrinsic evidence that the use of CsA in Jurkat cells reduced NF-κB activity.	Kronke et al. disclose "cloned leukemic T cells....induced with PHA..and PMA...for 6 hr in the presence or absence of CsA" (page 5215, second column). Siebenlist et al. teach "...a 2.5- and 4.5-h stimulation were done also in the presence of CsA.." (Figure 2, legend). Therefore, neither Kronke et al. nor Siebenlist et al. disclose reducing induced NF-κB activity. Likewise, as discussed above, neither Schmidt et al. nor Emmel et al. teach reducing induced NF-κB activity since neither Schmidt et al. nor Emmel et al. administer CsA after stimulation.
Thus, CsA is clearly recognized to have necessarily and inherently reduced NF-κB activity and resulting gene expression in those cells as called for by the instant claims.	It is inaccurate to state "thus, CsA is clearly recognized to have necessarily and inherently reduced NF-κB activity and resulting gene expression" since no reference either in the prior or post art demonstrated that the cells were induced prior to the administration of CsA. Hence there would be no NF-κB activity to be reduced.
Kronke and Siebenlist each disclosed the use of phytohaemaglutinin (PHA: 1ug/ml) and PMA (50ng/ml) to induce human T-cells (Jurkat cells).	Though both Kronke et al. and Siebenlist et al. disclose the use of PHA and PMA in their experiments, they do not teach the use of CsA to reduce PHA/PMA induced NF-κB activity.
Both references report that IL-2 (also known as "TCGF") gene transcription and/or expression was increased with administration of the NF-κB inducers phytohaemaglutinin (PHA) and phorbol 12-myristate-13-acetate (PMA), and that such induced IL-2 expression was reduced by CsA: The results of our study demonstrate that TCGF [IL-2] mRNA accumulation in induced Jurkat cells is diminished by CsA in a dose-dependent manner and that CsA acted by blocking TCGF mRNA transcription. Kronke at page 5217.	Kronke et al. and Siebenlist et al. do not report "such induced IL-2 expression was reduced by CsA." Kronke et al. state "1-hr preincubation, of concurrent administration of CsA resulting in almost complete inhibition of TCGF mRNA production. When added 4 hr after induction, CsA did not alter TCGF mRNA levels." (At page 5216, second column). Siebenlist et al. disclose "...the addition of CsA...completely prevented the formation of hypersensitive site III when added just 30 min before the addition of PHA and PMA (data not shown); under these

	conditions, no IL-2 message was observed." (page 3045, first column). Thus neither Kronke et al. nor Siebenlist et al. disclose reduction of induced IL-2 expression since both pretreated with CsA.
CsA at 1 ug/ml ... prevented IL-2 message induction. Siebenlist at page 3044, Fig. 2	Siebenlist et al. fully state "In the case of the Jurkat cells, a 2.5-and 4.5-h stimulation were done also in the presence of CsA at 1ug/ml (see text), which prevented IL-2 message induction" (page 3044, Fig.2). Siebenlist et al. teach that concurrent administration of CsA with the agent purported to induce NF-κB prevents or inhibits activation of NF-κB activity.
Using the same conditions and the same EMSA and CAT reporter assay formats as described in the instant '516 patent, the Schmidt reference clearly demonstrates that CsA as utilized in the Kronke and Siebenlist references reduced NF-κB activity in Jurkat cell cultures.	Kronke et al. administer CsA "either simultaneously with inducing agents or 1 to 4 hr prior to induction." (see Table 1, legend). Likewise, Siebenlist et al. conducted stimulation "in the presence of CsA" (see Figure 2, legend). Therefore, neither Kronke et al. nor Siebenlist et al. disclose "reduced NF-κB activity in Jurkat cell cultures" as alleged by the Examiner.
Schmidt reported, however, that there were inducers of NF-κB, which had different mechanisms of induction than PHA-induction, for which CsA had no effect. Specifically, Schmidt found that phorbol 12-myristate-13-acetate (PMA)-mediated induction of NF-κB activity was not affected by CsA.	Schmidt et al. report the differential effects of PMA and PHA on NFAT-1, not NF-κB activation. Specifically, Schmidt et al. disclose "...we have observed good activation of NFAT-1 with PHA alone but not with PMA alone (data not shown). Therefore the complete suppression of NFAT-1 in the presence of both PHA and PMA is consistent with the notion that CsA interferes with some necessary action(s) initiated by PHA but not with those initiated by PMA." (page 4038, first column).
The NF-κB CAT reporter assay confirmed this data, as depicted in Figure 4 ("inhibited the PHA-derived activation signal but not the PMA signal." Id. At 4039).	Schmidt et al. describe the use of HIV enhancer (κB-binding sites) in the CAT reporter assay depicted in Figure 4. Schmidt et al. do not equate the HIV enhancer with NF-κB.
Similarly, Emmel describes the effect of 1ug/ml of CsA on NF-κB activity in Jurkat cells that had been induced with both PHA and PMA.	Emmel et al. do not describe "induced" cells. Emmel et al. state "cells were stimulated for 8 hours with PHA...and PMA...with and without CsA." (Figure 1, legend). Therefore the cells were not

	induced prior to CsA administration.
As shown in Figure 1, 1ug/ml CsA (among other concentrations), reduced NF-κB activity ("NF-κB binding was reduced 10-20% in nuclear extracts of CsA-treated cells.". Emmel at 1618).	Figure 1 depicts inhibition of NF-κB activation resulting from the simultaneous treatment of cells with CsA and PHA/PMA.
Furthermore, a CAT assay confirmed these results, as shown in Figure 2d (Emmel at 1618) which demonstrates that CsA significantly reduced NF-κB activity at the same 1ug/ml concentration taught in Kronke and Sibenlist, as shown by the reduced expression of the NF-κB regulated CAT gene in those cells.	Emmel et al. provide no evidence showing that in Emmel et al.'s experiments a reduction in NF-κB activity was responsible for inhibiting the expression of any gene. Emmel et al.'s experiments showing prevention of activation of NF-κB (represented in Figure 1) are different from the experiments purporting to show inhibition of gene expression (represented in Figure 2). It is, therefore, incorrect for the Examiner to state that Emmel et al. "confirmed" CsA reduced NF-κB binding activity. Further, I do not understand the CAT gene to be regulated by NF-κB. The CAT gene is a bacterially derived gene and therefore is not endogenously regulated by NF-κB.
Thus, as discussed above, and in the Dr. Manolagas Declaration (Exhibit J: ¶¶ 25-32 of 90/007,503 request: incorporated by reference), Schmidt and Emmel confirm that Kronke and Siebenlist, which disclose the inhibitory effect of CsA on NF-κB activity in Jurkat cell cultures, anticipate claims 1-2, 5-6, 8-9, 20-21, 25-27, 29, 31-32, 36-38, 40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89, and 93-97 of the '516 patent (and as shown in Exhibit H-3 of 90/007,503 request: incorporated by reference.)	The Examiner acknowledges that CsA has an inhibitory effect on NF-κB activity consistent with the prior and post art which recites either a pretreatment of cells with CsA or concurrent administration of CsA with purported inducers. No reference discloses reducing induced NF-κB. Claims 1, 2, 5, 7, 20-21, 29, 31-32, 36-38, 40, 53-54 and 58-62 are not under review. Claims under review recite what they recite. As discussed in paragraph 12 above, Schmidt et al. and other have shown that CsA cannot reduce induced NF-κB activity.
The Siebenlist reference teaching of PHA and PMA stimulation done in the "presence of" CsA reads (chronologically) on addition of CsA either during or separately adding after PMA and PHA addition.	I do not understand what the Examiner means by "on addition of CsA either during or separately adding after PMA and PHA addition." I understand the protocol described in Siebenlist et al. in the legend

	of Figure 2 to mean that stimulation with PHA and PMA was "done in the presence of CsA" meaning that the cells were treated with all three reagents at the same time.

IV. PROTEIN KINASE C INHIBITORS

A. Express Anticipation Rejection based on Meichle et al. 1990 and Shirakawa et al. 1989

59. I have read the Examiner's comments in the July 6, 2007 Office Action pertaining to the article by Meichle et al. The Journal of Biological Chemistry (1990) 265(14):8339-8343 and Shirakawa et al. Molecular and Cellular Biology (1989) 9(6):2424-2430 and I respectfully disagree on numerous points summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 25-26 of July 6, 2007 Final Office Action)	
Meichle teaches the reduction of NF-κB activity in induced cells using agents that inhibit protein kinase C.	Meichle et al. do not teach the reduction of NF-κB activity in induced cells using agents that inhibit protein kinase C. Since Meichle et al. teach the administration of protein kinase inhibitors ">30 min before stimulation to allow equilibration and inhibition of enzymes" (page 8340, first column), there was no induced NF-κB activity to be reduced. The protein kinase inhibitors could, therefore, have only prevented or inhibited the activation of NF-κB by the subsequently administered agents
In addition, because Meichle used the HIV LTR in their experiments, this reference anticipates, or alternatively, makes obvious claims drawn to regulating expression of viral genes.	Meichle et al. use the "HIV-1 long terminal repeat enhancer sequence" (page 8340, first column), not the HIV LTR.
The instant claims are drawn to reducing NF-κB activity in eukaryotic, e.g. claims 1	Claims 1, 2 and 11 are not under review.

or 2, or mammalian cells (e.g. claim 11) to effect inhibited expression of a gene under transcriptional control of NF-κB.	
For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) to inhibit associated gene (viral gene such as HIV: claims 1-4) expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims 1-5 and 7 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling
Meichle analyzed various Protein Kinase C inhibitors and their effect on both PMA-(phorbol 12-myristate-13-acetate) and TNF-(tumor necrosis factor) induced NF-κB activity in eukaryotic Jurkat cells.	Meichle et al. analyzed the ability of various protein kinase inhibitors to prevent activation of NF-κB, not reduce induced activity. For example, Meichle et al. state "H7 in a dose-dependent manner reduced both TNF and PMA-induced PK-C activation in Jurkat cells..." (page 8341, first column).
Using an EMSA binding assay similar to that disclosed in the '516 patent, Meichle found that Protein Kinase C Inhibitor H8 reduced PMA-induced NF-κB activity in these cells (Fig. 3, lane 7).	It is inaccurate to state that Meichle et al. demonstrated that H8 "reduced PMA-induced NF-κB activity." The legend of Figure 3 clearly states that "Jurkat cells were preincubated for 30 min with protein kinase inhibitors H7 ... and H8... or a combination of H7 and H8 []. Cells were then stimulated for 20 min with TNF ... or PMA ..." (page 8342). The cells were not induced prior to the administration of the purported inhibitors since Meichle et al. stated that they were "preincubated" with inhibitors
Other inhibitors also were shown to reduce NF-κB activity, including Protein Kinase C Inhibitor H7 (Fig. 2B, lanes 3. vs. 5) and Staurosporine (Fig. 2B, lanes 6 and 7).	The legend of Figure 2 states that "prior to stimulation, cells were preincubated with H7 or staurosporine" (emphasis added, page 8341). This protocol would not allow for activation of NF-κB to occur and therefore this experiment does not demonstrate reduction of induced NF-κB activity.
Thus, Meichle teaches the use of Protein Kinase Inhibitor H8 (among others) to reduce NF-κB – mediated gene transcription by reducing NF-κB activity and reducing the binding of NF-κB to NF-κB binding sites.	It is inaccurate to state "Thus, Meichle et al. teach the use of Protein Kinase Inhibitor H8 (among others) to reduce NF-κB – mediated gene transcription by reducing NF-κB activity and reducing the binding of NF-κB to NF-κB binding sites." Meichle et al. do not demonstrate this since the

	protein kinase inhibitors were added to cells prior to the purported stimulus thereby preventing activation of NF-κB.
As such (and as shown in Exhibit G-2 of the 90/007,503 Request, hereby incorporated by reference) this reference expressly anticipates at least claims 1-2, 5-9, 20-21, 25-29, 31, 32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80-86, 88-89 and 93-97.	Exhibit G-2 relied on in the July 6, 2007 Final Office Action does not demonstrate anticipation of claims 6, 8, 9 and claims dependent thereon. I note that same statement is listed next to each of the different claims: "reducing NF-κB activity in cells" states "...Protein Kinase C Inhibitor H8 reduced PMA-induced NF-κB activity in these cells (Fig. 3, lane 7). Other inhibitors also reduced NF-κB activity, including Protein Kinase C Inhibitor H7 (Fig. 2B, lanes 3 vs. 5) and Staurosporine (Fig. 2B, lanes 6 and 7)." Exhibit G-2 mischaracterized the Meichle et al. reference. Meichle et al. explicitly state "H7 pretreatment of Jurkat cells impaired NF-κB activation by PMA." (page 8341, first column). Likewise, Meichle et al. state "At 150nM, staurosporine blocked PMA- but not TNF-induced activation of NF-κB..." (page 8341, second column). Thus, Meichle et al. teach only preventing or inhibiting NF-κB activity, not reducing induced NF-κB activity.
Additionally, because Meichle used a genetic construct comprising HIV LTR and NF-κB binding site, Meichle rendered claims 3, 4, 11, 42-43, 47-51, 106-107, 109-110, 114-117, 192-193 and 197-201 immediately envisaged (i.e. anticipated) or alternatively prima facie obvious in light of the fact that the HIV LTR promoter is responsible for regulating the expression of viral (HIV) genes.	Meichle et al. use the HIV LTR enhancer sequence, not the HIV LTR. I do not understand what "NF-κB binding site" the Examiner is referring to. Meichle et al. recite the use of the HIV ltR enhancer sequence only (see page 8340, second column, "Electrophoretic Mobility Shift Assays").

Examiner Statement (Examiner Response-page(s) 28 of July 6, 2007 Final Office Action)	
As taught by Meichle both TNF and PMA	I do not understand Meichle et al. to teach

stimulate NF-κB dependent viral (HIV-1) protein expression. See Meichle p. 8339, particularly 2nd column: "Release of NF-κB can be achieved by treating cells with phorbol esters, "; and p. 8340: "TNF, like PMA, strongly stimulated both HIV-1 and SV40 enhancer-driven chloramphenicol acetyltransferase gene expression in Jurkat cells."	that TNF and PMA stimulate viral protein expression as evidenced by the Examiner's citation. The experiments described only use the enhancer sequences from viral genes to drive expression of a bacterially-derived CAT reporter.

Examiner Statement (Examiner Response-page(s) 29 of July 6, 2007 Final Office Action)	
Accordingly, Meichle provides an enabled teaching of inhibiting NF-κB mediated viral (HIV or SV40) gene expression in induced human cells (Jurkat cells).	Meichle et al. do not teach the use of induced cells since the protein kinase inhibitors were always administered prior to the induction step. Further, I do not understand a cell that is transformed to express virally- and bacterially-derived components to be a human cell.

Examiner Statement (Rejection Summary-page(s) 31-32 of July 6, 2007 Final Office Action)	
Shirakawa teaches reduction of NF-κB activity in induced cells using agents that inhibit protein kinase C.	Shirakawa et al. recite first in the legend of Fig. 1 that "cells were in the presence... or absence... of ..H8 for 2h, washed, and then cultured with...IL-1 for 48 h" (page 2425). The legends of Table 1 and Figure 2 recite similar protocols. Therefore the administration of the protein kinase inhibitor prior to the induction step demonstrates prevention of activation, not reducing induced NF-κB activity.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1 or 2) or mammalian cells (e.g. claim 26) to effect inhibited expression of a gene under transcriptional control of NF-κB.	Claims 1, 2 and 26 are not under review.
For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) to inhibit associated gene (claims 1-2) expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claim 1-2, 5 and 7 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to

	require diminishing induced NF-κB mediated intracellular signaling
Analogous to Meichle discussed supra, Shirikawa performed similar tests with Protein Kinase C Inhibitor H8 on eukaryotic cells that had been induced with interleukin 1 (IL-1).	As stated above, Shirakawa et al. do not demonstrate an induction step followed by an inhibition step. Shirakawa et al. first treat with the purported inhibitor and follows with the agent purported to induce NF-κB activity.
The authors first demonstrated that IL-1 acted to induce NF-κB activity in 70Z/3 cells as demonstrated by the EMSA binding and CAT reporter assays (p. 2425 Fig. 1; p. 2426 Fig. 2).	I do not understand the use of 70Z/3 cells in the experiments described to be relevant to certain claims under review. 70Z/3 cells are murine pre-B lymphocytes and the claims specifically require the use of "human" cells.
The EMSA binding and CAT reporter assays then confirmed that Protein Kinase Inhibitor H8 reduced NF-κB activity and reduced the resulting CAT gene expression.	It is not accurate to state that the EMSA binding and CAT reporter assays "confirmed" that H8 reduced NF-κB activity. The EMSA binding and CAT reporter assays do not have a prior induction step to demonstrate that NF-κB activity was induced before administration of the inhibitors. Therefore, these assays only demonstrate that H8 prevented activation of NF-κB.
More particularly, the treatment of cells with H8 using EMSA resulted in "[t]he induction by IL-1 was abolished..." (p. 2426, Fig. 2A, lane 5)"; and using CAT "IL-1 induced κ immunoglobulin expression was markedly inhibited..." (p. 2425). These results were confirmed in a different cell line ("as was the case in 70Z/3 cells, NF-κB activation was markedly inhibited by H8 in YT cells (Fig. 2B, lane 9).	I do not understand the use of murine pre-B lymphocytes (70Z/3 cells) to be relevant to certain claims under review. Claims 71, 84 and 95 specifically require the method be carried out on "human cells."
Thus, Shirikawa teaches the use of Protein Kinase Inhibitor H8 (among others) to reduce NF-κB – mediated gene transcription by reducing NF-κB activity and reducing the binding of NF-κB to NF-κB binding sites.	First, the use of the CAT reporter assay does not evaluate the ability of H8 and other kinase inhibitors to affect NF-κB-mediated gene transcription since the CAT gene, which is bacterially-derived, is not under the control of NF-κB endogenously. Further, as stated above, the pretreatment of cells with H8 and other inhibitors only prevents activation of NF-κB, not reduce induced NF-κB activity.
As such (and as shown in Exhibit G-2 of	Claims 1-2, 5, 7, 21-21, 25-29, 31, 32, 36-

the 90/007,503 Request herein incorporated by reference) this reference expressly anticipates at least claims 1-2, 5-9, 20-21, 25-29, 31, 32, 36-40, 53-54, 58-62, 64-65, 69-73, 75-76, 80-86, 88-89 and 93-97	40, 53-54, 58-62 and 81 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling. Exhibit G-2 relied on in the July 6, 2007 Final Office Action lists the following same statement next to each of claims 6, 8, 9 and claims dependent thereon: "The induction by IL-1 was abolished by treatment of cells with H8 (Fig. 2A, lane 5)." This statement reaffirms that Shirakawa et al. pretreat cells with protein kinase inhibitors to prevent or inhibit activation of NF-κB activity. Thus, even Exhibit G-2 acknowledged that Shirakawa et al. do not teach reducing induced NF-κB activity.

Examiner Statement (Examiner Response-page(s) 33 of July 6, 2007 Final Office Action)	
Further it is noted that the patentee has overlooked the Shirakawa teaching of the ability of protein kinase inhibitors, such as H-8, to reduce IL-1 induced NF-κB activity and *inhibit* protein expression *resulting* from NF-κB activation. See Shirakawa p. 2425, right column under "Results".	On page 2425, Shirakawa et al state "IL-induced κ immunoglobulin expression was markedly inhibited." However, these experiments, represented in Figure 1 and Table 1, state that cells were "incubated in the presence...or absence...of 10μM H8 for 2 h, washed, and then cultured with...or without..IL-1 for 48h" (Figure 1, legend) or "were incubated in the presence or absence of ..IL-1...with or without polymyxin B" (Table 1, legend) or "were incubated with...IL-1...in the presence or absence of polymyxin B...palmitoylcarnitine...or cholorpromazine.." (Table 1, legend). Considering all cells were either pretreated with inhibitors or exposed to inhibitors concurrently with IL-1, this clearly means that protein kinase inhibitors might only

	prevent activation of NF-κB, which is consistent with the entire Shirakawa et al. document and my understanding.

60. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Meichle et al. teach administration of protein kinase C inhibitors to reduce NF-κB activity. I find no such teaching Meichle et al. or Shirakawa et al. Meichle et al. administer protein kinase inhibitors prior to the administration of agent purported to activate NF-κB as described, e.g. on page 8340, first column, "protein kinase inhibitors were added >30 min before stimulation to allow equilibration and inhibition of the enzymes." Further, Meichle et al. state "H7 pretreatment of Jurkat cells impaired NFκB activation by PMA...In contrast, H7 did not inhibit NF-κB activation by TNF. The use of the protein kinase inhibitor staurosporine revealed similar results. At 150nM, staurosporine blocked PMA- but not TNF-induced activation of NF-κB..." (page 8341, first column). Inhibiting activation is a method different from diminishing induced NF-κB activity. Thus, Meichle et al. do not teach administration of protein kinase inhibitors to diminish induced NF-κB-mediated intracellular signaling as recited in claim 6.

61. Shirakawa et al. also disclose that "cells were incubated in the presence....or absence...of 10μM H8 for 2 h, washed and then cultured with...or without...1.2 x 10^{-11}M IL-1 for 48h) (see Figure 1 legend, page 2425). Similar treatment protocols are described for the experiments presented in the remaining figures. Inhibiting activation is a method different from diminishing induced NF-κB activity. Thus, Shirakawa et al. do not teach administration of protein kinase inhibitors to diminish induced NF-κB-mediated intracellular signaling as recited in claim 6.

62. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Meichle et al. do not demonstrate that the administration of protein kinase inhibitors modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. Meichle et al. present data demonstrating that the protein kinase inhibitors H7 and staurosporine can prevent stimulation of a cell by PMA (Figure 2, page 8341). Preventing stimulation is a method different from modifying effects of external influences that induce NF-κB activity. Thus, Meichle et al. do not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

63. Shirakawa et al. also do not demonstrate that the administration of protein kinase inhibitors modified the effects of external influences which induce NF-κB-mediated intracellular signaling. Shirakawa et al. present data demonstrating that in YT cells pretreated with H8 for 2 hours prior to stimulation with LPS "NF-κB activation was markedly inhibited" (page 2426, first column). Inhibiting activation is a method different from modifying effects of external influences that induce NF-κB activity. Thus, Shirakawa et al. cannot teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

64. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Meichle et al. do not provide any experiment demonstrating a protein kinase C inhibitor-mediated reduction in the level of expression of any

gene which is activated by extracellular influences which induce NF-κB-mediated intracellular signaling. First, Meichle et al. only teach pretreatment with a protein kinase C inhibitor to prevent activation. Meichle et al. provide EMSA assays describing the effect of protein kinase inhibitor pretreatment on the ability of NF-κB to bind to oligonucleotides encompassing "two tandemly arranged NF-κB binding sites of the HIV-1 enhancer" (see page 8340).

65. Further, the NF-κB binding site of the HIV-1 enhancer is not a gene endogenously regulated by NF-κB. Whether protein kinase C can even prevent activation of NF-κB at all is put into question by Figure 3 of Meichle et al. which demonstrates that "TNF induces NFκB in the presence of protein kinase inhibitors H7 and H8" (page 8342, Figure 3 legend). Regardless, preventing activation is a different method from reducing the level of expression of genes which are activated. Thus, Meichle et al. do not teach reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

66. Likewise, Shirakawa et al. do not provide any experiment demonstrating protein kinase C inhibitor-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Shirakawa et al. do demonstrate that "As was the case in 70Z/3 cells, NF-κB activation was markedly inhibited by H8 in YT cells ...(page 2426, first column)." However, preventing activation is a different method from modifying the effects of extracellular influences that induce NF-κB-mediated intracellular signaling. Thus, Shirakawa et al. do not teach reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

67. Claims 64, 65 and 69 further limit claim 6, claims 75, 76 and 80 further limit claim 8, and claims 88, 89 and 93 further limit claim 9 by requiring that the reduction of induced NF-κB activity occurs by interference at a specific segment in the NF-κB pathway. Meichle et al. do not disclose how a protein kinase inhibitor could reduce induced NF-κB activity. I note that Exhibit G-2, relied on in the July 6, 2007 Final Office Action, has the following identical statement next to each of claims 64, 65, 69, 75, 76, 80, 88, 89 and 90: "Meichle found, using a EMSA (binding) assay similar to that disclosed in the '516 patent, that Protein Kinase C Inhibitor H8 reduced PMA-induced NF-κB activity in these cells (Fig. 3, lane 7). Other inhibitors also reduced NF-κB activity, including Protein Kinase C inhibitor H7 (Fig 2B, lanes 3 vs. 5) and Staurosproine (Fig. 2B, lanes 6 and 7)." The statements in Exhibit G-2 do not explain how the protein kinase inhibitors operate, but rather describe only the observed result. Claims 64-69, 75-80 and 88-93, however, specifically require reduction of induced NF-κB activity by interfering at a specific segment of the NF-κB pathway.

68. Likewise, Shirakawa et al. do not disclose any mechanism by which a protein kinase inhibitor could reduce induced NF-κB activity. I note that Exhibit G-2, relied on in the July 6, 2007 Final Office Action, has the following identical statement next to each of claims 64, 65, 69, 75, 76, 80, 88, 89 and 90: "In an EMSA assay, Shirakawa found that "The induction by IL-1 was abolished by treatment of cells with H8 (Fig. 2A, lane 5). The statement in Exhibit G-2 does not explain how the protein kinase inhibitors operate, but rather describes only the observed result. The statement in Exhibit G-2 also reaffirms that "induction" was abolished. Claims 64, 65, 69, 75, 76, 80, 88, 89 and 90, however, specifically require reduction of induced NF-κB activity by interfering at a specific segment of the NF-κB pathway.

69. Further, Meichle et al. state "PK-C has not only been implicated in mediating TNF activation of the transcription factor AP-1 [], but is also likely to be involved in the activation of the IκB:NF-κB complex" (page 8339, second column). Likewise, Shirakawa et al. disclose "PKA and PKC can activate NF-κB" and they hypothesize "that IκB is the target of phosphorylation; the phosphorylated IκB would presumably have a decreased ability to bind NF-kB." (page 2428, second column). If I were to understand that the administration of protein kinase inhibitors has any effect on NF-κB activity, and I am not certain that it does, the only possible conclusion given the results of Meichle et al. and Shirakawa et al. is that the administration of protein kinase inhibitors prevents activation of NF-κB by preventing the phospohrylation of IκB. Neither Meichle et al. nor Shirakawa et al. disclose the administration of protein kinase inhibitors reduced induced NF-κB activity.

70. I have carefully reviewed Meichle et al. and Shirakawa et al. and determined that the experimental procedures described in Meichle et al. and Shirakawa et al. do not enable one skilled in the art to reproduce the results presented. For example, the "highly purified recombinant TNF" used in these studies was obtained from a source not readily available to the public. The TNF was produced by Genentech and provided by Dr. G. Adolf (page 8340, first column). A member of the public cannot expect to obtain the same results with any form of recombinant TNF. Further the modifications that may have been made to the recombinant TNF are not articulated in Meichle et al. and therefore one would not reasonably know if all batches of recombinant TNF are equivalent. Likewise, in Shirakawa et al., the origin of critical reagents is unclear. For example, "partially purified PKC from human peripheral blood neutrophils" were "kindly provided by S. Sozzani and L. McPhail" (page 2425, first column). This reagent, which is used in the experiments described in Figures 5 and 6, would not be readily available to the

general public and therefore could not enable one to reproduce experiments described. Even if one could obtain this reagent, it is generated from cellular extracts and its concentration is listed in units (U). Variation of preparation of the cellular extracts can influence protein concentration which would render the "units" meaningless. Likewise, the origin of the kinase inhibitor used, H8, is not indicated anywhere in the text. Consequently, neither Meichle et al. nor Shirakawa et al. provide sufficient instruction to enable one of skill in the art to reproduce the experiments described.

V. Vitamin D/Calcitriol

A. Inherent Anticipation Rejection Based on Tsoukas et al., Manolagas et al., Rigby et al. I, and Lemire et al. I and II as evidenced by Yu et al. and the Declaration of Dr. Manolagas

71. I understand that the Examiner has alleged that Tsoukas et al., Manolagas et al., Rigby et al. I, Lemire et al. I and Lemire et al. II each inherently anticipate claims 6, 64-73, 8, 75-80, 82, 84 9, and 88-97. I understand the Examiner's position to be that the method being claimed in claims 6, 8 and 9 and claims dependent thereon is described in Tsoukas et al., Manolagas et al., Rigby et al. I, Lemire et al. I and Lemire et al. II based on Yu et al. and the undated Declaration of Dr. Manolagas. I respectfully disagree. I have reviewed the claims, Tsoukas et al., Manolagas et al., Rigby et al. I, Lemire et al. I, Lemire et al. II, Yu et al. and the Manolagas Declaration and determined that none of these references disclose the method of the claims under review. In the sections which follow, I first present my observations of Tsoukas et al., Manolagas et al., Rigby et al. I, Lemire et al. I, Lemire et al. II and then present my observation of the non-prior art references, Yu et al. and the Manolagas Declaration, which the July 6, 2007 Final Office Action purports explains these references.

72. I have read the Examiner's comments in the July 6, 2007 Final Office Action pertaining to the articles by Tsoukas et al. Science (1984) 224:1438-1440; Manolagas et al. Journal of Clinical Endocrinology and Metabolism (19860 63(2):394-400; Rigby et al. Journal of Clinical Investigation (1984) 74:1451-1455; Lemire et al. Journal of Clinical Investigation (1984) 74:657-661; Lemire et al. The Journal of Immunology (1985) 134(5):3032-3035 as evidenced by Yu et al. Proc. Natl. Acad. Sci. USA (1995) 92:10990-10994 and the Manolagas Declaration and I respectfully disagree of numerous points summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 78-80 of July 6, 2007 Final Office Action)	
Tsoukas, Manolagas, Lemire I, Lemire II or Rigby I teach administration of calcitriol to humans, which as is shown from the teachings of Yu, inherently reduces NF-κB activity and thus would inhibit expression of genes whose transcription is regulated by NF-κB activity.	Tsoukas et al., Manolagas et al, Lemire et al I, Lemire et al II, Rigby et al I and Yu et al do not teach the administration of calcitriol to humans. I do not find any experiment in any of the cited references wherein calcitriol is administered to humans.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1, 2 or 5) or mammalian cells (e.g. claim 26) to effect inhibited expression of a gene under transcriptional control of NF-κB.	Claims 1, 2, 5 and 26 are not under review.
For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) or decreasing the level of NF-κB not bound in an NF-κB –IκB complex (claim 20) to inhibit associated gene expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims 5, 7 and 20 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized to require diminishing induced NF-κB mediated intracellular signaling.
Calcitriol (1, 25-dihydroxyvitamin D_3) is the active form of Vitamin D that has been administered to humans for decades. There are numerous prior art publications that report the study of calcitriol in various human cell cultures, including human Jurkat (T-cell line) leukemic cells (citations omitted) and human peripheral blood monocyte ("PBM") cells, including	I do not understand the administration of calcitriol to humans to be equivalent to the treatment of cultured cells with calcitriol.

Tsoukas (PBM), Manolagas (PBM) Lemire I, Lemire II and Rigby I.	
Tsoukas and Manolagas teach that the administration of calcitriol (at least 10^{-8} M) in PBM cells reduced IL-2 activity; a result which was confirmed by Lemire I, Rigby I and Lemire II (e.g.Lemire II at 3034 demonstrated that calcitriol exhibited "a dramatic and specific reduction of IL-2 production by activated PBM...").	Tsoukas et al. and Manolagas et al. teach the co-administration of calcitriol with an agent purported to induce NF-κB activity (see Tsoukas et al, page 1438, Figure 1 legend; see Manolagas et al., page 399, Figure 5 legend). Therefore, "administration of calcitriol...in PBM cells reduced IL-2 activity" is inaccurate. The presence of calcitriol during administration of PHA prevented activation of NF-κB.
Independent studies show that IL-2 expression is regulated, at least in part, by NF-κB. See Manolagas Declaration at page 8, ¶ 15 and documents cited therein.	The Manolagas Declaration mischaracterizes the results Schmidt et al., cited to support the conclusion that IL-2 expression is regulated, at least in part, by NF-κB. Schmidt et al. do not provide any experiment wherein the expression of IL-2 is evaluated.
Yu et al. under the same conditions utilized in Tsoukas and Manolagas (i.e. calcitriol application to PHA activated PBM cells) found that calcitriol reduced NF-κB as well as NF-κB – regulated gene expression in PBM cell cultures.	Discussed below at length.
Yu described the use of Electrophoretic Mobility Shift Assays ("EMSAs" as in Ex. 15 of the instant '516 patent) and the NF-κB binding sequence of human IL-6 promoter to demonstrate (by EMSA) that calcitriol administration "caused a significant reduction in the DNA-protein complex, as evidenced by the decrease in the intensity of this band (lane 4)" (see Yu at 10993 and Fig. 5).	Yu et al. disclose a method wherein "PBMCs were incubated without or with PHA alone or in the presence of 10^{-8}M of $1,25(OH)_2D_3$ for 72 hr." (emphasis added, Figure 4, legend). Yu et al. also state that the experiment represented in Figure 5 was conducted to evaluate "...the inhibiting effect of $1,25(OH)_2D_3$ of NF-κB expression..." (emphasis added, page 10993, first column). Thus, Yu et al. disclose inhibiting or preventing activation of NF-κB activity, not reducing induced NF-κB activity.
This data was confirmed using Western blot analyses (Figs. 1 and 3), which showed that calcitriol added to PHA-activated cells (as taught in Tsoukas and Manolagas) "caused a significant decrease in the expression of p50 [subunit of NF-κB] at all time points examined". Id. At 10991-2.	Tsoukas et al. and Manolagas et al. teach the co-administration of calcitriol with an agent purported to induce NF-κB activity (see Tsoukas et al., page 1438, Figure 1 legend; see Manolagas et al., page 399, Figure 5 legend). Therefore, it is inaccurate to state that Tsoukas et al. and

	Manolagas et al. teach "PHA-activated cells."
In an analogous manner in human Jurkat leukemic cells transfected with a CAT gene regulated by NF-κB and activated with PHA, Yu found that calcitriol cell application resulted in reduced NF-κB gene expression. See Yu at 10993.	Yu et al. disclose in Figure 6 "Jurkat cells were transfected with the p(NF-κB)$_4$-CAT plasmid....After transfection cells were cultured in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$ for 48 hr and stimulated with PHA for the last 8 hr of the 48-hour period." (emphasis added, page 10993). I do not understand this to describe "reduced NF-κB gene expression" since CAT is not a gene regulated by NF-κB and the pretreatment of the transformed cells with calcitriol prevented activation of NF-κB.
Although the mechanism may not have been known at the time of the Tsoukas, Manolagas, Lemire I, Lemire II and Rigby I publications, the Yu reference evidence proved that the administration of calcitriol by these prior art references necessarily and inherently reduced NF-κB activity in induced human cells (PBM/Jurkat) and reduced expression of NF-κB regulated proteins (IL-2). Thus, these prior art references anticipate at least claims 1-2, 5-6, 8-9, 20-21, 25-27, 29, 31-32, 36-38, 40, 53-54, 58-62, 64-65, 69-73, 75-76, 80, 82, 84, 88-89, and 93-97 of the '516 patent as shown on an element-by-element basis in Exhibit H-4, H5 and H6 of the 90/007,503 request (incorporated by reference)	Discussed at length below

Examiner Statement (Examiner Response-page(s) 80 of July 6, 2007 Final Office Action)	
Additionally, as discussed supra, the instant invention encompasses administering an NF-κB inhibitor prior to, with or subsequent to administering an NF-κb activating compound.	I do not understand the instant invention to encompass administration as stated by the Examiner. The invention of the claims under review encompasses only reducing induced NF-κB activity. The now claimed invention does not encompass pretreating cells with an inhibitor prior to exposure to a compound purported to activate NF-κB as this would prevent induction of NF-κB

	and would be outside the scope of the invention.
Further, in contrast to patentee's argument, the prior art references establish the ability of calcitriol to "inhibit" protein (e.g. IL-2) production in an "activated" mammalian or eukaryotic cell(s) whether conicubated or subsequently contacted with an already activated cell as in Tsoukas 1984 (Abstract, page 1438 and Table 1) which teaches that vitamin D3 (calcitriol) "inhibited IL2" production in PHA "induced" human peripheral blood mononuclear cells (PBM) leucocytes when coincubated with PHA.	First, Tsoukas et al. do not teach the use of "induced" human PBMCs. In the legend of Figure 1, Tsoukas et al. clearly state that the PBMCs were "cultured with various concentrations of $1,25(OH)_2D_3$ in 1ml of medium RPMI 1640 supplemented with PHA (1%)..." (page 1438).

Examiner Statement (Examiner Response-page(s) 81 of July 6, 2007 Final Office Action)	
Similarly in Lemire II (see pages 3032 under "Cell culture conditions" to top of page. 3033 (top)) the cells are first activated with PWA, concanavalin A or PHA followed by the addition of 10^{-8}M $1,25(OH)_2$ which serves in the assays to suppress Ig production (see page 3033 under "Results and Discussion" and page 3034 right column: "...dramatic and specific reduction of IL-2 production by activated PBM incubated with $1,25(OH)_2D_3$).	I do not understand Lemire et al. II to describe an activation step followed by an inhibition step. For clarification, the legend of Figure 1 specifically recites "1,25-$(OH)_2D_3$-pretreatment" (page 3033). Further, the legend of Figure 2 recites " T_H and T_S cells (1 x 10^8 cells/mL) from three different human subjects were cultured with PWM and Con A, respectively, for 2 days with or without 10^{-8} M $1,25(OH)_2D_3$." (page 3034).

Examiner Statement (Examiner Response-page 84(s) of July 6, 2007 Final Office Action)	
It is further noted that Yu (1995) teaches the ability of calictriol to inhibit the presence of NF-κB (p50 and p105) when added simultaneously with PHA or 24, 48, 64, 68 or 70 hours following addition of PHA.	Yu et al. state that "when $1,25(OH)_2D_3$ was added into the culture for the last 4 hr (68 hr following PHA activation) or the last 2 hr (70 hr following PHA activation) of the 72-hr culture period, there was no discernable effect."(Page 10992, first column).

73. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Tsoukas et al., Manolagas et al., Lemire et al. I and II teach administration of calcitriol to reduce NF-κB activity. I find no such teaching in these prior art references. Tsoukas et al., Manolagas et al., Lemire et al. I and II administer calcitriol to cells at the same time as the inducers (see Tsoukas et al., page 1438 Figure 1 legend; Manolagas et al., page 395 second column; Lemire et al. I, page 658 first column; and Lemire et al. II, page 3032 bottom of second column) to inhibit activation of NF-κB. Inhibiting activation is a method different from diminishing induced NF-κB activity. Therefore the act of administering calcitriol concurrently with the purported inducer of NF-κB activity does not meet the requirement of reducing induced NF-κB activity.

74. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Tsoukas et al., Manolagas et al., Lemire et al. I and II do not teach or disclose any experiment wherein the administration of calcitriol modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. Therefore, Tsoukas et al., Manolagas et al., Lemire et al. I and II do not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as required by claim 8.

75. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Tsoukas et al., Manolagas et al., Lemire et al. I and II do

not teach or provide any experiment demonstrating a calcitriol-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Therefore, Tsoukas et al., Manolagas et al., Lemire et al. I and II do not teach reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

76. Claims 64, 65 and 69 further limit claim 6, 75, 76 and 80 further limit claim 8, and 88, 89 and 93 further limit claim 9 by requiring that the reduction of induced NF-κB activity occurs by a specific mechanism. Tsoukas et al., Manolagas et al., Lemire et al. I and II do not disclose a mechanism by which calcitriol could be reducing induced NF-κB activity. I note that Exhibits H-4 and H-5, relied on in the July 6,2007 Final Office Action recites the identical statement next to each of claims 64, 65, 69, 75, 76, 80, 88, 89 and 90: "Inherent. See Yu." The Examiner is relying on the extrinsic reference Yu et al. to describe the mechanism of reducing induced NF-κB activity therefore I do not understand Tsoukas et al., Manolagas et al., Lemire et al. I and II to explain by which mechanism calcitriol operates. Claims 64, 65, 69, 75, 76, 80, 88, 89 and 90 specifically require reduction of induced NF-κB activity <u>by</u> a specific mechanism which is not described in Tsoukas et al., Manolagas et al., Lemire et al. I or II.

Inherency: Rigby et al. I as evidenced by Yu et al.

77. Rigby et al. I show calcitriol-mediated reduction in cellular proliferation and, in Figure 4, a reduction in the level of IL-2 production mediated by calcitriol over the course of 18 hours. A noticeable reduction in IL-2 production is observed at 12 hours post-calcitriol treatment and, at 18 hours, no IL-2 is observed (Figure 4). The production of IL-2 is thought to be regulated, at least in part, by NF-κB. Both Schmidt et al. and Emmel et al., both references of record, have

demonstrated that factors, other than NF-κB, have a role in regulating IL-2 production. Schmidt et al. acknowledge that "NFAT-1-binding site...has been implicated directly in playing a major role during transcriptional activation of IL-2" (emphasis added, page 4037, first column). Likewise, Emmel et al. state "NF-AT, AP-3 and to a lesser extent NF-κB....appear to be important in the transcriptional activation of genes for interleukin-2.." (page 1617, abstract). Additionally, cellular proliferation is a process regulated by many factors, not simply NF-κB. Thus, cellular proliferation and IL-2 production are not necessarily indicative of NF-κB activity. In Rigby et al. I, the cellular proliferation and IL-2 production results are not attributable to reduction of induced NF-κB activity as explained by Yu et al., Takeuchi et al. and Alroy et al.

78. Yu et al. evaluated the effects of calcitriol on IL-2 production and cellular proliferation noting that:

> NF-κB enhances the expression of IL-2 and the IL-2 receptor, two molecules critical for lymphocyte proliferation. On the other hand, $1,25(OH)_2D_3$ inhibits IL-2 production and lymphocyte proliferation, raising the possibility that the antiproliferative effects of $1,25(OH)_2D_3$ could have been mediated via inhibition of NF-κB. However, the time courses of the effects of $1,25(OH)_2D_3$ on NF-κB expression and lymphocyte proliferation were different. Specifically, whereas $1,25(OH)_2D_3$ could inhibit cell proliferation only when it was added in the first 24 hr following activation $1, 25(OH)_2D_3$ decreased p50 and 105 levels when added to the culture as late as 64 hr following addition of the activating agent. This suggests that the antiproliferative effect of $1,25(OH)_2D_3$ is not the result of it effects on NF-κB" (page 10994, first column).

Yu et al. demonstrated that the addition of calictriol 48 hours after activation with PHA inhibits the transcription of the NF-κB subunits p50 and p105 as disclosed in Figure 1. On page 10992, Yu et al. teach that "PBMCs were activated for 72 hr with PHA, and $1,25(OH)_2D_3$ was added either simultaneously with addition of PHA or 24, 48, 64, 68, or 70 hr following addition of PHA (Fig. 3). The inhibiting effect of $1,25(OH)_2D_3$ on p50 and p105 was apparent when the hormone was added simultaneously with PHA or 24, 48, or 64 hr following addition of PHA." Yu et al.,

therefore, demonstrated that whatever effect $1,25(OH)_2D_3$ has on IL-2 production, that effect is different from reducing induced NF-κB.

79. The time course of effects of $1,25(OH)_2D_3$ observed by Yu et al. is notably different from the time course observed by Rigby et al. I. Rigby et al. I demonstrated in Figure 4, that the IL-2 production effects of calcitriol on PHA-stimulated PBMs occurred within 18 hours, and after 18 hours no IL-2 production remained to inhibit. According to Yu et al., however, the only effect of $1,25(OH)_2D_3$, ie. its effect on transcription of p50 and p105 is detected after 24 hours.

80. The effect of calictriol on proliferation and IL-2 production in Rigby et al. I occurs too rapidly to be attributed to the effect of $1,25(OH)_2D_3$ on the transcription of the NF-κB subunits p50 and p105. As described in the '516 patent, column 16, lines 22-28, "NF-κB is initially located in the cytoplasm in a form unable to bind DNA because it is complexed with IκB. Various inducers then cause an alteration in IκB allowing NF-κB to be released from the complex. Free NF-κB then travels to the nucleus and interacts with its DNA recognition sites to facilitate gene transcription." Thus, transcription of p50 and p105 genes is not needed for induction of NF-κB activity. Consequently, based on the kinetics of synthesis of the p50 subunit and rapid activation of NF-κB targets, it is unlikely that NF-κB can be activated by an inducing substance much sooner than if transcription of the p50 and p105 subunits were needed.

81. Takuechi et al. and Alroy et al. offer further insight. Both demonstrate that IL-2 production can be activated by any one of four factors: NF-κB, NFAT, AP-1 and OCT-3 (see Takuechi et al. at page 209). Based on Yu et al.'s teaching that the only effect of $1\alpha,25(OH)_2D_3$ relative to NF-κB is its inhibition of transcription of p105 and p50, it is evident that the results

observed in Rigby et al. I in terms of cellular proliferation and IL-2 production could not have involved only reducing induced NF-κB activity.

82. Consequently, I understand that in Rigby et al. I a factor other than NF-κB must have been affected resulting in the reduced transcription of IL-2. My conclusion is further supported by the fact that a reduction of p50 and p105 would not lead to reduced NF-κB activity because p65/RelA alone is sufficient to promote transcription of IL-2 (see eg. Nishiyama et al., page 704, Figure 4, a copy of which is attached hereto as **Exhibit 10**). Therefore, I understand that the results in Rigby et al. I cannot show that calcitriol reduces induced NF-κB activty as required by claims 6, 8 and 9.

83. Further, I understand that the experiments presented in the prior art, which either entail simultaneous administration of calcitriol with PHA (as in Tsoukas et al., Manolagas et al., Lemire et al. I and Lemire et al. II) or are unable to demonstrate inhibition of the active subunit of NF-κB (as in Rigby et al. I as evidenced by Yu et al.) do not disclose a mechanism of reducing induced NF-κB activity as recited in claims 64, 75 and 88, which recite "NF-κB activity is reduced by decreasing the level of NF-κB not bound in an NF-κB:IκB complex" or the mechanism recited by claims 65, 76 and 89 wherein "NF-κB activity is reduced by inhibiting the passage of NF-κB into the nucleus of the cells" or the mechanism recited by claims 69, 80 and 93 which require "reducing binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB."

Non-reproducibility of Experiments

84. Further, I do not understand the methods presented in the cited prior art to reasonably enable one to perform the experiments and obtain the same results described in Tsoukas et al., Manolagas et al., Rigby et al. I, Lemire et al. I or II. For many of the experiments cited in the prior art, critical reagents would not be available to the public as there were either obtained as "gifts" or their source is not identified. For instance, in Lemire et al. I and II, the vitamin D analogs were "generously provided by Dr. John Babacok" and $1,25(OH)_2D_3$ and 24R,25 dihydroxyvitamin D were "provided by Dr. Milan Uskokovic" (Lemire et al. I, page 658, first column; Lemire et al. II, page 3033, first column). Rigby et al. I list similar sources for the vitamin D used in their study (see page 1451, second column "Methods"). Likewise, the anti-TAC antibody used in the studies presented in Lemire et al. II was obtained from Dr. T.A. Waldmann (Lemire et al. II, page 3033, first column).

85. Manolagas et al. has similar problems wherein multiple reagents including the vitamin D, anti-Tac antibody and monoclonal antibody to MHC Class II (Ia) molecules were provided by private sources not available to the public (page 395, first column). Tsoukas et al. fail to disclose the sources of any reagents, such as $1,25(OH)_2D_3$ and PHA used in the experiments presented. Consequently, failure to provide sources or use reagents available to the general public would make it impossible for one to repeat the experiments described and achieve the same results.

86. Further, the procedure described in Lemire et al. I and II is not clear as to how the reagents were added. Lemire et al. I simply state that "at the initiation of the culture period, pokeweed mitogen (PWM) (Sigma Chemical Co.), phytohemagglutinin-P (PHA-P)(Difco Laboratories, Detroit, MI) ....was added to a second group of cell cultures.....Vitamin D sterol were solubilized in absolute ethanol and added to culture medium..." (page 658, first column). Lemire et al. II describes a similar protocol wherein "pokeweed mitogen (PWM)...or

phytohemaggultinin-P...were added at the initiation of the culture period....Authentic $1,25(OH)_2D_3$....was added as a 0.025% solution to the culture medium..." (page 3032, second column). I do not understand either of these protocols to state that calcitriol was added after incubation with the purported stimulus nor do I find the procedure clearly described to enable one of skill in the art to determine the order in which the reagents were added. As I read the methods, I understand it to mean that the reagents were added at the same time and therefore would not allow for the necessary induced NF-κB activity as required by the claimed method.

VI. Endogenous Glucocorticoids

A. Inherent Anticipation Rejection Based on Art Relating to Glucocorticoids (Goodman and Gilman's as evidenced by Baldwin et al. I, Auphan et al., Scheinman et al. I and II, Mukaida et al. and Padgett et al.)

87. I have read the Examiner's comments in the July 6, 2007 Final Office Action regarding the experiment presented Goodman and Gilman's The Pharmocological Basis of Therapeutics, Macmillan Publishing Co., Inc. 1980, pages 1466-1496; Baldwin et al. Annu. Rev. Immunol. 1996 14:649-681; Auphan et al. Science (1995) 270:286-290; Scheinman et al. Mol. Cell. Biol. (1995) 15:943-953; Scheinman et al. Science (1995) 270:283-286; Mukaida et al. J. Biol. Chem (1994) 269:13289-13295; and Padgett et al. Trends in Immunology (2003) 24(8):444-448 relating to glucocorticoids and I respectfully disagree on numerous points as summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 94-97 of July 6, 2007 Final Office Action)	
Goodman & Gilman teach the naturally occurring endogenous production and release of glucocorticoids (cortisol) in response to many different types of environmental stressors which inherently	I do not understand how production of endogenous glucocorticoids in response to environmental stressors is analogous to reducing induced NF-κB activity. First, nothing of record indicates which of the

results in the reduction of NF-κB activity that acts to inhibit expression, in a eukaryotic cell, of cytokines whose transcription is regulated by NF-κB.	"many different types of environmental stressors" induce NF-κB. Second, nothing of record shows which endogenous glucocorticoid has any effect, much less a reducing effect on those environmental stressors which might induce NF-κB.
In this respect, the NF-κB inhibiting evidence provided by the Baldwin I, Auphan, Scheinman I/II and the Mukaida documents, regarding the synthetically administered steroid dexamethasone (DEX) has been found to be analogous with what occurs upon release of the endogenous cortisol glucocorticoid as evidenced by Padgett.	Dexamethasone is not an endogenous glucocortioid produced by the body. I do not understand Baldwin et al. I, Auphan et al., Scheinmann et al. I and II and the Mukaida et al. documents to provide a correlation between the use of dexamethasone and the endogenous production of different glucocorticoids in response to stress.
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1, 2 or 5) or mammalian cells (e.g. claim26) to effect inhibited expression of a gene under transcriptional control of NF-κB. For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) or decreasing the level of NF-κB not bound in an NF-κB-I-κB complex (claim 20) to inhibit associated gene expression of a cytokine protein (claim 5) in a eukaryotic cell. The instant claims broadly encompass the natural process in which an animal responds to environmental stressors by increasing production of endogenous glucocorticoids (cortisol, cortisone, hydrocortisone, corticosterone, aldosterone: see e.g. page 1473 Table 63-2 that are secreted into the bloodstream. Increased glucocorticoids are produced in response to many different types of stressors including "agonal state, severe infections, surgery, parturition, cold, exercise, and emotional stress" (Goodman and Gilman, 1469, "Example of effective Stimuli of Secretion"). These elevated quantities of endogenous glucocorticoids are known to "prevent or suppress the inflammatory response that takes place as a consequence of the hypersensitivity reactions" (Goodman and Gilman, page 1479, left	The instant claims are drawn to reducing induced NF-κB activity. I do not understand how production of endogenous glucocorticoids in response to environmental stressors is analogous to reducing induced NF-κB activity. First, nothing of record indicates which of the "many different types of environmental stressors" induce NF-κB. Second, nothing of record shows which endogenous glucocorticoid has any effect, much less a reducing effect on those environmental stressors which might induce NF-κB.

column, "Immune Responses").	
However, it has been known since the mid-1990's, that glucocorticoids effect their immunosuppression by reducing NF-κB activity by preventing NF-κB from binding to the appropriate sites on the DNA and by increasing the transcription, expression and/or release of IκB, thereby reducing the amount of activated NF-κB that can translocate into the nucleus.	Inflammation is not solely associated with induction of NF-κB. The effects of glucocorticoids to modulate inflammation are not necessarily attributable to inhibiting NF-κB as discussed below.
In this regard, several publications cited by Dr. Baldwin in his 1996 review article (Baldwin I), including Auphan, Scheinman I, Scheinman II and Mukaida, in addition to the Padgett document, provide extrinsic evidence that endogenously produced glucocorticoids, such as cortisol, are recognized to necessarily and inherently reduce NF-κB activity thus inhibiting cytokine production, in vivo, upon the body's release of glucocorticoids in response to external stumuli, such as stress, as described in Goodman and Gilman.	Auphan et al., Scheinman et al. I and II and Mukaida et al. do not demonstrate that glucocorticoids produced in the body will inhibit NF-κB activation to modulate inflammation in the same manner as the synthetic glucocorticoid, dexamethasone, inhibits the activation of NF-κB in cultured cells. Scheinman et al. I and II and Mukaida et al. do not teach any in vivo administration of dexamethasone. Only Auphan et al. describe administration of dexamethosone (concurrently with an agent purported to activate NF-κB) in mice. However, Auphan et al. do not demonstrate that the administration of dexamethsone can reduce induced NF-κB. Auphan et al. state "Injection of mice with anti-CD3 caused degradation of thymic IκBα, but simultaneous administration of DEX resulted in IκBα levels similar to those in untreated animals." (page 288, middle column).
A) Extrinsic Evidence of Inherency as Provided by Auphan	
Auphan describes methods for reducing NF-κB activity in an induced leukemic T-cell line.	Auphan et al. do not describe an "induced leukemic T-cell line." Auphan et al. describe administration of dexamethasone concurrently with TPA thereby preventing activation of NF-κB, not reducing induced NF-κB activity (see legend, Figure 1).
First, Auphan induced human leukemic T cells (Jurkat strain) or murine T cells (FJ8.1) with TPA and used EMSA, as described in the '516 Baltimore patent, to demonstrate NF-κB activity.	Auphan et al. did not "first" induce human leukemic T cells (Jurkat strain) or murine T cells (FJ8.1). In the legend of Fig. 1, Auphan et al. state that FJ8.1 cells were "incubated for 24 hours with either medium alone (UN) or with 4μM

	ionomycin (I) and TPA (T) (100ng/ml) in the absence or presence of 1μM DEX." Auphan et al. describe a similar treatment for transfected Jurkat cells in the legend of Fig. 2.
Auphan then exposed the cells to 10^{-6} M dexamethasone. Id. Auphan reports that dexamethasone clearly reduced NF-κB activity: Electrophoretic mobility shift assays (EMSAs) revealed that both AP-1 and NF-κB DNA binding activities were elevated in nuclear extracts of activated [FJ8.1] cells (fig. 1C).	Auphan et al. did not "then" expose the cells to 10^{-6} M dexamethasone. Dexamethasone was administered at the same time as the agents purported to induce NF-κB activity. Auphan et al. clearly state that "DEX inhibited induction of NF-κB binding activity..." (page 287, middle column).
DEX inhibited induction of NF-κB binding activity and reduced the amount of AP-1 binding activity DEX also inhibited induction of NF-κB in mouse T lymphocytes in vivo (Fig. 1D).	Inhibition of induction of NF-κB is a method different from reducing induced NF-κB activity as recited in the claims under review.
Inhibition of NF-κB activiation by DEX... was also observed in a Jurkat human T cell leukemia line stably transfected with a GR expression vector (Fig. 2A)... Inhibition of NF-κB activity was also observed in cells stimulated by either 12-0-tetradecanoryl-phorbal 13-acetate (TPA) alone or by tumor necrosis factor (TNF-α). Id. (citations omitted).	Inhibition of induction of NF-κB is a method different from reducing induced NF-κB activity as recited in the claims under review.
As Dr. Baldwin noted, Auphan demonstrates that glucocorticoids "involves the transcriptional activation of the I-κBα gene" in these human leukemic cells and therefore, "by upregulating I-κBα protein levels, function to block nuclear translocation of NF-κB and DNA binding." Baldwin at 671. As discussed below, Dr. Baldwin's own results, as reported in the Scheinman II document discussed below, confirms this mechanism for glucocorticoid action.	Baldwin et al. I reference Auphan et al. in the section of the review discussing "Inhibitors of NF-κB Activation" (emphasis added, page 671). Thus, Baldwin et al. I acknowledge that glucocorticoids prevent or inhibit activation of NF-κB. Further, other references cited by Baldwin et al. I in this section, namely Mukaida et al., Scheinman et al. I and II and Auphan et al. all pretreat with dexamethsone, reaffirming that dexamethasone prevents activation of NF-κB.
B) Extrinsic Evidence of Inherency as	

Provided by Scheinman I & II	
Scheinman I and Sccheinman II confirm that dexamethasone, as used in the prior art, reduced NF-κB activity.	Scheinman et al. I and II demonstrate that dexamethasone prevents induction of NF-κB activity, not reduces NF-κB activity since both studies administer dexamethasone concurrently with the agents purported to stimulate NF-κB.
In particular, Scheinman I evaluated the effect of 10^{-7} M dexamethasone on NF-κB activity in human epithelial (HeLa) cell cultures. Scheinman I at 944. NF-κB activity was measured in at least two assay formats disclosed in the Baltimore '516 patent (EMSA and reporter assays) as well as Western blot immunological assays.	Sheinman et al. I disclose "HeLa cells were pretreated with DEX for 16 h and then treated with TNF-α for 1 h..." (page 949, first column).
Dexamethasone was shown (Figs 1A and B; Fig. 4; and Fig. 7) to reduce endogenous NF-κB activity in cells ("pretreatment with DEX resulted in a profound loss of TNF-α induced NF-κB as well as p50 homodimer (KBF1) gel shift activity (Fig. 7B, lane 3). Similar data were obtained by treating cells with IL-1 (data not shown)". ID at 949	The statement cited by the Examiner clearly states "pretreatment with DEX resulted in a profound loss of TNF-α induced NF-κB" which I understand to mean that the pretreatment with dexamethasone prevented activation of NF-κB rather than reduced induced NF-κB activity.
Scheinman I concluded that dexamethasone reduces NF-κB activity by two distinct avenues: We show that GR can physically interact with NF-κB subunits and also block their ability to bind DNA. In addition, we present new data showing that dexamethasone (DEX) treatment of HeLa cells causes a significant reduction in nuclear p65 protein levels.	Scheinman et al. I demonstrate that "glucorticoids block activation of endogenous NFκB activity" (page 949, first column).
Thus, glucocorticoids are also able to inhibit NF-κB activity by a novel mechanism involving a block of cytokine-induced nuclear translocation.	Scheinman et al. I also teach "Glucocorticoids block activation of endogenous NF-κB activity"(emphasis added, page 949, first column).
With EMSA analysis, we found that DEX treatment caused a marked reduction in the ability of NF-κB/Rel subunits to bind DNA despite the presence of equal amounts of NF-κB subunit protein in the EMSA DNA-binding reaction mixtures (Fig. 5, EMSA). Id at 944, 948	As is stated in the legend of Figure 5, "Cultures were either treated with DEX immediately after transfection (+) or left untreated (-)..." (page 948). I understand this description to mean that DEX prevents activation of NF-κB thereby resulting in a comparative reduction in binding as compared to the untreated samples.
Similarly, the Scheinman II reference	Scheinman et al. II describe cell cultures as

demonstrates that dexamethasone, at the same 10^{-7} M concentration as Scheinman I, reduces NF-κB activity as called for by the claims that are the subject of this reexamination request.	"pretreated for 12 hours with 10^{-7} M dexamethasone and stimulated for 1 hour with TNF-α (1ng/ml)" (emphasis added, Figure 1, legend). The legends of the other figures describe similar treatment protocols. Therefore, Scheinmann et al. II do not teach reducing induced NF-κB activity.
Scheinman II confirmed the Auphan conclusion that dexamethasone inhibits TNF α induced NF-κB activity, at least in part, by inducing the transcription of the IκB-α gene: Together, these data indicate that DEX treatment induces the transcription of the IκB-α gene. This induction results in the increased syntheses of the IκB-α protein. This increase in protein synthesis leads to the rapid turnover of the IκB-α protein associated with preexisting NF-κB complexes. In the presence of an activator such as TNF α, newly released NF-κB reassociates with the DEX-induced IκBα and thus reduces the amount of NF-κB translocating to the nucleus. Scheinman II at 286.	Scheinman et al. II do not present evidence wherein induced NF-κB activity is reduced by dexamethasone treatment. The administration of dexamethasone "induces IκBα gene transcription" which I understand to further be a method different from reducing induced NF-κB activity. IκBα is a NF-κB target gene which is inhibited not activated. Sheinman et al. II provide no experimental evidence that dexamethasone can increase IκBα transcription and such increase can reduce induced NF-κB activity.
C) Extrinsic Evidence of Inherency as Provided by Mukaida	
Mukaida observed that IL-8 production was mediated by NF-κB in all cells they previously examined. Mukaida at 13284.	Mukaida et al. spans pages 13289 to 13295. Therefore, I do not understand which cells the Examiner is asserting where Mukaida et al. evaluated IL-8 production.
In those cells, dexamethasone inhibited NF-κB-regulated IL-8 production by more than 60% at concentrations equal to and higher than 10^{-8}M. id at 13290.	Mukaida et al. teach "Cells were then stimulated with 10ng/ml human rIL-1α in the presence of various concentrations of dexamethasone (DEX) for 24 h." (emphasis added, page 13291, Figure 1 legend). Thus Mukaida et al. teach administration of DEX and the agent purported to stimulate NF-κB activity results in preventing NF-κB activation not reducing induced NF-κB activity.
D) Extrinsic Evidence of Inherency as Provided by Padgett	
Consistent with the Scheinman II and Auphan references, Padgett teaches that the	I do not understand how production of endogenous glucocorticoids in response to

body responds to external stressors by release of glucocorticoid hormones including cortisol, which bind glucocorticoid receptors expressed on a variety of immune cells to interfere with NF-κB function, resulting in reduced cytokine production. See Abstract: p.445 (right column) to p. 446; footnotes 32 and 33; and p. 447 under "conclusions."	environmental stressors is analogous to reducing induced NF-κB activity. First, nothing of record indicates which of the "many different types of environmental stressors" induce NF-κB. Second, nothing of record shows which endogenous glucocorticoid has any effect, much less a reducing effect on those environmental stressors which might induce NF-κB. Padgett et al. cite Scheinman et al. II and Auphan et al. which, as discussed above, do not teach the administration of dexamethasone to reduce induced NF-κb activity. Further Padgett et al. state "stress hormones inhibit...the production of proinflammatory cytokines and chenokines..." (emphasis added, page 445, first column). Padgett et al. discuss multiple stress hormones including glucocorticoids and catecholamines. Thus, Padgett et al. do not teach that glucocorticoids specifically reduce induced NF-κB activity.
Thus, endogenous production of glucocorticoids in response to external body stimuli as described in Goodman and Gilman is now recognized as necessarily inherently reducing NF-κB activity as called for by the subject claims of the Baltimore '516 patent. See also '7503 Exhibit H8 (incorporated by reference) application to claims regarding exogenous glucocorticoid administration.	Padgett et al. state "the GR undoubtedly interferes with the function of other transcriptional regulators. It is thought that protein-protein interactions similar to those described for GR-NF-κB are involved with GC inhibition of activator protein-1 (AP-1) and nuclear factor of activated T lymphocytes (NF-AT)." (page 447). Because glucorticoids can affect multiple transcription factors it is inaccurate to state that glucocorticoids "necessarily inherently" reduced NF-κB activity. Finally, Exhibit H-8 relied on in the July 6, 2007 Final Office Action is not relevant as it analyzes references which have been withdrawn and no longer cited in the July 6, 2007 Final Office Action.
Further, in contrast to the patentee's argument, all of the evidentiary references teach inhibition of NF-κB activated cells utilizing a glucocorticoid (cortisol or dexamethasone) regardless of sequence of	It is inaccurate to state that "all" of the evidentiary references teach inhibition of NF-κB "activated" cells. As discussed above, the evidentiary references do no describe "activated" cells since

activation and inhibition.	dexamethasone was administered either prior to or with the agent purported to induce NF-κB activity.

88. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that Goodman and Gilman teach administration of glucocorticoids to reduce NF-κB activity as evidenced by the post-filing art references. I find no such teaching in Goodman and Gilman or the post art references. Goodman and Gilman describe the production of glucocorticoids by the body in response to "agonal state, severe infections, surgery, parturition, cold, exercise and emotional stress" (Goodman and Gilman's page 1469, second column) while the post-filing art references of Auphan et al., Scheinman et al. I and II and Mukaida et al. describe the administration of a synthetic glucocorticoid, dexamethasone, either prior to treatment with an inducer (see Auphan et al., Figure 2 legend; "Jurkat cells...were incubated with TPA...DEX...a combination of TPA plus DEX, or no further addition"; Scheinman et al. I, page 944, "HeLa cells were allowed to recover for 4 h after removal of the crystals and then treated with DEX...TNF-α (2ng/ml) or IL-1 (10ng/ml) was then added 20 h later..."; Scheinman et al. II, Figure 1 legend, "HeLa cultures were pretreated for 12 hours with 10^{-7}M DEX and stimulated 1 hour with TNF-α (1ng/ml)"; Mukaida et al., Figure 1 legend "Cells were then stimulated with 10ng/ml human rIL-2α in the presence of various concentrations of dexamethasone (DEX) for 24 h"). Inhibiting activation is a method different from diminishing induced NF-κB activity. Thus, Auphan et al., Mukaida et al., Sheinman et al. I and II do not teach administration of dexamethasone to diminish induced NF-κB-mediated intracellular signaling as recited in claim 6.

89. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." Neither Goodman and Gilman nor the cited post-filing art references demonstrate that the administration of a glucocorticoid modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. The post-filing art references describe experiments wherein, as is stated by Auphan et al. on page 287, first column, "GCs are potent inhibitors of NF-κB activation." Preventing stimulation is a method different from modifying effects of external influences that induce NF-κB activity. Thus, Goodman and Gilman and the post art reference do not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as required by claim 8.

90. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Goodman and Gilman do not provide any experiment demonstrating a glucocorticoid-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling. Likewise, Auphan et al. evaluate IL-2 production using cells treated simultaneously with ionomycin, TPA and DEX (see Figure 1 legend). As discussed above in paragraph 87, the post-art references describe either pretreatment with DEX or simultaneous administration of DEX with agents purported to induce NF-κB activity.

91. Likewise, Scheinman et al. I describe experiments using eukaryotic cells transformed with plasmids comprising the "major histocompability complex (MHC) class I $H\text{-}2k^b$ NF-κB site

cloned upstream of a minimal *fos* promoter chloramphenicol acetyltransferase (CAT) expression vector (63) and the luciferase reporter construct pGL2-Basic" (page 944, first column). I do not understand the CAT or luciferase genes to be endogenously regulated by NF-κB. Mukaida et al. describe similar experiments using "CAT expression vectors harboring the 5'-flanking region of the IL-8 gene" (page 13290, first column). I do not understand the CAT or luciferase genes to be endogenously regulated by NF-κB. Additionally, Scheinman et al. II do not describe any experiment involving a gene regulated by NF-κB. Thus, the cited references cannot teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

92. Claims 71, 84 and 95 are drawn to the practice of the method on "human cells." Auphan et al., Mukaida et al. and Scheinman et al. II do not disclose the use of a "human cell." Auphan et al. use a Jurkat cell "stably transfected with an expression vector encoding a rat GR and expressing 20,000 receptors per cell" (Page 287, Figure 2 legend). Mukaida et al. and Scheinman et al. II use transformed human glioblastoma and Jurkat T cells, a human T cell line, respectively. The glioblastoma cell described by Mukaida et al. has been manipulated to express, at high levels, "CAT expression vectors harboring the 5'-flanking region of the IL-8 gene" (page 13290, first column). Further, the Jurkat T cell line used by Scheinman et al. II has been manipulated to express, at high levels plasmids comprising the "major histocompability complex (MHC) class I *H-2k*^{b} NF-κB site cloned upstream of a minimal *fos* promoter chloramphenicol acetyltransferase (CAT) expression vector (63) and the luciferase reporter construct pGL2-Basic" (page 944, first column) . In my expert opinion I don't find any of these cells, which express bacterially-derived reporter genes, to be considered a "human cell."

93. Claims 64, 65 and 69 further limit claim 6, 75, 76 and 80 further limit claim 8, and 88, 89 and 93 further limit claim 9 by requiring that the reduction of induced NF-κB activity occurs by interference at a specific segment in the NF-κB pathway. Goodman and Gilman do not disclose how glucocorticoids could reduce induced NF-κB activity. Of the post art references, Scheinman et al. I propose that "GR can physically interact with NF-κB subunits and also block their ability to bind DNA. In addition...dexamethasone (DEX) treatment of HeLa cells causes a significant reduction in nuclear 665 protein levels. Thus, glucocorticoids are also able to inhibit NF-κB activity by a novel mechanism involving a block of cytokine-induced nuclear translocation" (page 944, first column). Likewise, Auphan et al. suggest that "the simplest explanation of the current results is that newly synthesized IκBα translocates to the nucleus, where, as shown in vitro, it can sequester free NF-κB and thereby promote net dissociation of DNA-bound NF-κB. However, the experiments presented in Scheinman et al. I and Auphan et al. were conducted using cells treated with dexamethasone before stimulation or concurrently with stimulation therefore it is unclear whether dexamethasone would have the same effect on cells stimulated first and then treated with dexamethasone. The results described above do not explain how dexamethasone operates, but rather describes only the observed result. Claims 64, 65, 69, 75, 76, 80, 88, 89 and 90, however, specifically require reduction of <u>induced</u> NF-κB activity <u>by</u> interfering at a specific segment of the NF-κB pathway.

94. Further, I understand that glucocorticoids can be produced in response to many stimuli and do not specifically target and reduce induced NF-κB activity. There is no evidence provided that NF-κB activity is induced in response to the above-described stimulators of glucocorticoids. Furthermore, there is no extrinsic evidence presented that demonstrates localization of glucocorticoids to the sites of inflammation. As stated in Goodman and Gilman, "cortisol and

presumably other anti-inflammatory steroids are to be found in inflamed tissue, although they are not selectively concentrated there" (page 1480, first column). Even if glucocorticoids are localized to the sites of inflammation, there is no evidence presented that indicates that inflammation mediated by induced NF-κB activity can be reduced by glucocorticoids.

95. Finally, there is no experiment described by the post art references wherein glucocorticoids localize to the sites of inflammation and reduced induced NF-κB activity thereby resolving inflammation. In fact, there is experimental evidence to the contrary. Greten et al., a copy of which it attached as **Exhibit 11**, developed a transgenic mouse deficient for gastrointestinal NF-κB activation, referred to as "*villin-Cre/Ikkβ* $^{F/\Delta}$" mice. Upon stimulation with 2.5% DSS, "higher levels of mRNAs for proinflammatory proteins, such as TNFα, IL-1β, ICAM, IL-6" were detected in the *villin-Cre/Ikkβ* $^{F/\Delta}$ mice (Greten et al., page 288, second column). This result clearly demonstrates that an inability to activate NF-κB *in vivo* does not prevent inflammation, indicating that factors other than NF-κB can mediate the inflammatory response. Therefore, I disagree with the conclusion that administration or production of glucocorticoids would inherently reduce induced NF-κB activity. I further find that Goodman and Gilman could not teach that the production or administration of glucocorticoids reduces induced NF-κB activity as evidenced by the extrinsic references since those references failed to first induce NF-B, failed to provide any *in vivo* data and, in certain cases, utilized a cell system irrelevant to the scope of the pending claims.

VII. 5-ASA

A. Inherent anticipation rejection based on Dew et al. 1983 as evidenced by Baldwin et al. II, Bantel et al., Yan et al. and the David Baltimore Declaration

96. I have read the Examiner's remarks in the July 6, 2007 Office Action pertaining to the article Dew et al. British Medical Journal (1983) 287:23-24 in view of Baldwin et al. J. Clin. Invest. (1991) 107:63-80, Bantel et al. Amer. J. Gastroenterology (2000) 287:3452, Yan et al. J. Biol. Chem. (1999) 274:366631-36 and I disagree on several points summarized in the table below.

Examiner Statement (Summary-page(s) 90-91 of July 6, 2007 Final Office Action)	
Dew teaches administration of 5-ASA for the treatment of ulcerative colitis in humans,	I do not understand Dew et al. to teach the administration of 5-ASA for the "treatment of ulcerative colitis in humans" as Dew et al. clearly state on page 23 that all patients "were in remission with ulcerative colitis."
which inherently reduced NF-κB activity in the cells of the patients, by a mechanism including phosphorylation of IκB.	It is incorrect the state that 5-ASA "inherently reduced NF-κB activity in the cells of the patients." Dew et al. do not evaluate NF-κB activity in this study nor does Dew et al. indicate that patients undergoing remission of ulcerative colitis have induced NF-κB activity. Finally, Dew et al. disclose that "seven of the 32 patients taking 5-amino salicyclic acid (22%)...relapsed" (page 23, second column).
The instant claims are drawn to reducing NF-κB activity in eukaryotic (eg claims 1, 2 or 5) or mammalian cells (eg claim 26) to effect inhibited expression of a gene under transcriptional control of NF-κB.	Claims 1, 2, 5 and 26 are not under review.
For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) or decreasing the level of NF-κB not bound in an NF-κB –IκB complex (claim 20) to inhibit associated gene expression of a cytokine protein (claim 5) in a eukaryotic cell.	Claims 5, 7 and 20 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized as requiring diminishing induced NF-κB-mediated intracellular signaling.
Dew teaches the administration (oral delayed release of up to 4.4 g/day) of 5-aminosalicyclic acid (5-ASA) to humans	Dew et al. do not teach the administration of 5-ASA "for the treatment of ulcerative colitis." Dew et al. state "we determined

for the treatment of ulcerative colitis (an inflammatory bowel disease). Ulcerative colitis is recognized as being associated with NF-κB activation. See Baldwin II at Table 1.	whether higher doses of 5-amnio salicyclic acid were more effective in preventing relapse..." (page 23, first column). Preventing relapse and treating active ulcerative colitis are not equivalent.
Bantel conducted in vivo tests (tissue sample immunostaining using p65 NF-κB subunit antibody before/after 5-ASA administration) on human ulcerative colitis patients administered the same 5-ASA formulation (tradename MESALAZINE) in the same amounts (from 1.7-4.5 g/day) as in the Dew reference and reported that 5-ASA administration reduced NF-κB activity in the cells of these patients. Bantel at 3453	Discussed below concerning formulation differences
Specifically, Bantel found that (1) NF-κB activity is increased in patients with ulcerative colitis, and (2) therapeutic administration of 5-ASA (as in Dew) effectively reduced NF-κB activity in these patients. Id. At 3454-55.	Discussed below concerning formulation differences
Accordingly, Bantel concluded that "5-ASA is a potent inhibitor of NF-κB activation in vivo," at dosage levels and under the same conditions taught in Dew. Id at 3456.	Discussed below concerning formulation differences
Thus, Dew's administration of 5-ASA for ulcerative colitis inherently and necessarily reduces NF-κB activity in human cells.	Discussed below concerning formulation differences
Moreover, during prosecution of the Baltimore 08/464,364 application, while referring to the Yan reference (cited above), Dr. Baltimore in his declaration admitted that 5-ASA reduces NF-κB activity as in the instant '516 patent claims: "treatment of cells with such compounds as ... 5-aminosalicyclic acid...inhibits NF-κB mediated gene expression by a mechanism which includes inhibition of phosphorylation of I-κB proteins" (which is the naturally occurring NF-κB inhibitor). See David Baltimore declaration ¶9.	I do not understand Yan et al. to support the statement referenced in the Baltimore Declaration. Yan et al. disclose cell as "pretreated with 5-ASA...for 30 min followed by TNFα" (page 36632, first column). Thus, Yan et al. only demonstrate the administration of 5-ASA prevents or inhibits activation of NF-κB and does not teach reducing induced NF-κB activity.
Accordingly, the prior art Dew administration of 5-ASA necessarily and inherently reduced NF-κB activity and, thus anticipated at least claims 1-2, 5-6, 8-	Claim 1, 2, 5, 7, 20-27, 29, 31-38, 40, and 53-62 are not under review. Further Dew et al. do not inherently teach reduced NF-κB activity since not only were the patients

9, 20-27, 29, 31-38, 40,53-62, 64-73, 75-80, 82, 84, and 88-97 as shown on an element-by-element basis in Exhibit H-7 (herein incorporated by reference) provided by the requester in the 90/007,503 proceeding.	not undergoing active ulcerative colitis, but Dew et al. do not teach that inactive ulcerative colitis is characterized by cells with elevated cytokine levels. Further, Exhibit H-7 relied on in the July 6, 2007 Final Office Action does not show anticipation of the claims under review for the reasons discussed below.

Examiner Statement (Summary-page(s) 92 of July 6, 2007 Final Office Action)	
Further, patentee fails to appreciate the Dew reference suggestion that high doses of 5-ASA, effective in maintaining remission in ulcerative colitis, "might be of value in *treating* ulcerative colitis."	The full sentence of Dew et al. to which the July 6, 2007 Final Office Action refers is: "It is possible that the higher doses might be of value in treating acute colitis." This sentence reflects Dew et al.'s speculation and is not a teaching to practice the method of the claims under review, nor a teaching of how to do so, nor a teaching that doing so would be successful. I do not understand this sentence of Dew et al. to provide sufficient information for practicing a method of reducing induced NF-κB activity.

Inherency

97. Claim 6 and the claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged the Dew et al. teach administration of 5-ASA to reduce induced NF-κB activity in patients undergoing remission of ulcerative colitis. I find no such teaching in Dew et al. Contrary to the July 6, 2007 Final Office Action and Exhibit H-7 cited, which next to claim 6 has the statement "Administration of oral dosages of 2.4-4.4 g/day of 5-ASA to human patients with ulcerative colitis...," Dew et al. describe no patient with ulcerative colitis and states that "all were in remission with ulcerative colitis or proctitis and had passed three or less stools daily without blood or slime during the previous month" (emphasis added, page 23). Therefore, since Dew et

al. do not demonstrate patients with active ulcerative colitis, there is no induced NF-κB activity to be reduced. Thus Dew et al. do not teach administration of 5-ASA to diminish induced NF-κB-mediated intracellular signaling as recited in claim 6.

98. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on eukaryotic cells, which external influences induce NF-κB-mediated intracellular signaling." Dew et al. do not demonstrate that the administration of 5-ASA to patients with inactive ulcerative colitis modifies the effects of external influences which induce NF-κB-mediated intracellular signaling. Therefore, Dew et al. do not teach modification of the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 8.

99. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Dew et al. do not disclose any experiment demonstrating a 5-ASA-mediated reduction in the level of expression of genes activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

100. Furthermore, Dew et al. do not disclose any NF-κB regulated gene whose activity was modified by the administration of 5-ASA. Therefore, Dew et al. do not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

101. Further, the Examiner alleged that Yamamoto et al. teach patients in remission with ulcerative colitis still have elevated cytokine levels, indicating NF-κB is active. I disagree.

Yamamoto et al. only evaluate patients "who had been diagnosed with endoscopically and histologically confirmed UC" (page 590, first column). Thus, Yamamoto et al. did not evaluate healthy volunteers to determine normal cytokine levels. Further, both Braegger et al. and Mitsuyama et al., copies of each are attached as **Exhibit 12 and 13**, analyze patients with inactive disease, those who are in "remission" and control patients to show patients in remission have cytokine levels similar to control patients. Specifically, Braegger et al. state "in patients with inactive disease, either as a result of surgery or treatment with steroids, the concentration of stool TNF alpha fell to those of control" (abstract). Likewise, Figure 1 of Mitsuyama et al. demonstrates that the colonic levels of IL-8 in patients with inactive ulcerative colitis are no different for those observed in the control patients. The Examiner has acknowledged on page 92 of the Final Office Action, that TNF and IL-8 are known to be regulated by NF-κB. Therefore, I disagree with the statement that patients with inactive ulcerative colitis maintained elevated levels of NF-κB regulated cytokines and therefore, I understand that the patients in Dew et al. would not have had elevated cytokine levels which would indicate the presence of activated cells. Consequently, Bantel et al. and Yan et al., as cited by the Examiner would not be relevant to Dew et al. since both Bantel et al. and Yan et al. describe patients who have active ulcerative colitis.

102. Regardless of how "remission" in Dew et al. is interpreted, Dew et al. cannot be shown to reduce induced NF-κB activity. The reference purported to explain Dew et al., namely Bantel et al., only shows that 5-ASA can prevent induction of NF-κB. I note that Exhibit H-7, relied on in the July 6, 2007 Final Office Action, has the following identical statement next to each of claims 64-69, 75-80 and 88-93: "5-ASA treatment in patients with ulcerative colitis "almost completely abrogated NF-κB activation"." Bantel et al. disclose that "5-ASA is a potent inhibitor of NF-κB

activation.." (emphasis added, page 3456, second column). Thus if "remission" in Dew et al. is understood to indicate induced NF-κB activity, Bantel et al. cannot explain what happened in Dew et al.; on the other hand, if "remission" is understood to mean no NF-κB activity, Dew et al. did not practice the method of the claims under review.

103. Claims 64-69 further limit claims 6, 75-80 further limit claim 8, and 88-93 further limit claim 9 by requiring that the reduction of induced NF-κB activity occurs by interfering at a specific segment of the NF-κB pathway. Neither Dew et al. nor the post art, Bantel et al., disclose that 5-ASA reduces induced NF-κB by interfering at a specific segment of the NF-κB pathway as recited by claims 64-69, 75-80 and 88-93.

104. The Examiner has alleged on page 93 of the July 6, 2007 Final Office Action that the formulation differences between the 5-ASA administered by Dew et al. and the 5-ASA administered by Bantel et al. would have no effect on the outcome obtained by Dew et al. as compared to the outcome obtained by Bantel et al.. I understand the formulation differences to have a profound effect. Dew et al. utilize 5-ASA coated with Eudragit-S, which releases at pH≥ 7.0 while Bantel et al. use 5-ASA coated in Eudragit-L which releases at pH≥ 6.0. Steinhart et al. elaborate on the significance of these differences (a copy is attached as **Exhibit 14**). On page 1395, Steinhart et al. state "pH 7-dependent mesalazine has a greater therapeutic benefit, with a lower NNT compared with pH 6-dependent and controlled-release mesalazine. In addition, the pH7-dependent mesalazine has a statistically significant advantage over placebo, whereas neither the pH 6-dependent and controlled-release mesalazines do when each is analysed against placebo. It is surprising that such an apparently minor difference in delivery system might have a measurable clinical effect, but this review is based on published evidence." Not only does

Steinhart et al. teach that the differences in formulation have significant impact on the efficacy of the drug, I would not understand that had Dew et al. used the same formulation as Bantel et al., the outcome of the Dew et al. study would have been the same as that reported. Since I understand that Bantel et al. conducted their study with a completely different formulation of 5-ASA, I disagree that the result obtained in Bantel et al. could explain the result obtained by Dew et al..

105. Finally, as I stated previously in paragraph 95 above, inflammation is not due only to NF-κB activity. For example, the production of proinflammatory cytokines in the gastrointestinal tract of the *villin-Cre/Ikkβ*$^{F/\Delta}$ mice and resulting colonic inflammation occurred in the absence of functional NF-κB (see paragraph 94). Therefore I disagree that the modulation of inflammation necessarily involves a reduction of induced NF-κB activity.

VIII. N-ACETYL-L-CYSTEINE

A. Express Anticipation Rejection based on Staal et al. 1990

106. I have read the Examiner's comments in the July 6, 2007 Final Office Action regarding the article Staal et al. Proc. Natl. Acad. Sci. (1990) 87:9943-9947 and I disagree on several points summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 111-112 of July 6, 2007 Final Office Action)	
Staal et l. (see Abstract; page 9945; Figures 4-5) disclose a method of inhibiting TNF-α by blocking NF-κB activation in mammalian cells (e.g. Jurkat cells) by administration of N-acetyl-L-cysteine (NAC).	Staal et al describe experiments where cells were simultaneously treated with NAC and TNF-α. Therefore, Staal et al. cannot show a method to reduce intracellular signaling caused by TNF-α, as recited by claim 18.
Instant claim 18 recites: A method for	Claim 18 is correctly recited.

reducing Interleukin-1 or Tumor Necrosis Factor-α activity in mammalian cells comprising reducing NF-κB activity in the cells so as to reduce intracellular signaling caused by interleukin-1 or Tumor Necrosis Factor-α in the cells.	
Staal teaches a method of inhibiting TNF-α activation and, thus, signaling, in mammalian cells by reducing NF-κB activity.	Staal et al. do not disclose "a method of inhibiting TNF-α activation and, thus signaling, in mammalian cells by reducing NF-κB activity." According to Staal et al. "findings presented here demonstrating that NAC blocks cytokine-stimulated NF-κB activation." (emphasis added, page 9947, first column).
Specifically, Staal discloses inhibiting TNF-α stimulation of 293.27.2 and Jurkat cells by selectively blocking NF-κB activation by administration of N-acetyl-L-cysteine (NAC). See abstract, and Fig. 5.	Staal et al. do show inhibiting TNF-α stimulation of cells using simultaneous administration of NAC.
Reduction of intracellular TNF-α signaling is demonstrated by decreased expression of a beta-galactosidase reporter gene in Jurkat cells, which are mammalian (human) in origin. See Fig. 4. accordingly, claim 18 is anticipated. Instant claim 182, depending from claim 18, further recites that reducing NF-κB activity comprises reducing binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB.	The Jurkat cell line referred to is the "Jurkat tri-κB" cell. These cells have been transformed to express a construct of unknown origin, since the construction details of said plasmid are listed as a "personal communication" (page 9943, second column). I do not understand these cells in Staal et al. to be "mammalian" or "human."
Instant claim 182, depending from claim 18, further recites that reducing NF-κB activity comprises reducing binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB. Staal anticipates claim 182 by teaching that high thiol levels (resulting from NAC administration) inhibit NF-κB activation by preventing phosphorylation of I-κB, since phosphorylation of I-κB is necessary for dissociation of NF-κB from its complex with I-κB	Inhibiting "activation" of NF-κB is different from reducing induced NF-κB activity.
Since NAC administration keeps NF-κB complexed to its naturally occurring inhibitor I-κB, NF-κB is unavailable for binding NF-κB recognition sites on gene that are transcriptionally regulated by NF-	I find no description of an experiment in Staal et al. that discloses "NAC administration keeps NF-κB complexed to its naturally occurring inhibitor I-κB, NF-κB is unavailable for binding NF-κB

κB.	recognition sites on gene that are transcriptionally regulated by NF-κB."
Thus, instant claim 182 is anticipated since NAC-mediated inactivation of NF-κB described in Staal inherently and necessarily results in reduced NF-κB binding to its recognition sites.	Staal et al. do not describe NAC-mediated inactivation of NF-κB. Staal et al. teach co-administration of PMA, TNF-α, and NAC (page 9943, second column) thus NAC did not mediate "inactivation of NF-κB" rather NAC prevented activation of NF-κB. Staal et al. clearly state that "NAC inhibits stimulation with PMA considerably more effectively than it inhibits stimulation with TNF-α." (page 9944, second column). Further, claim 182 recites "reducing NF-κB activity comprising reducing binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB." Staal et al. do not disclose any gene regulated by NF-κB since Staal et al. utilizes the "Jurkat tri-κB cells" and 293.27.2 cells transformed to express the "HIV LTR fused to bacterial β-galactosidase." (see 9943, second column). Neither the tri-κB construct (whose origin is unknown) or the β-galactosidase gene are regulated by NF-κB.
Instant claims 183-185, depending from claim 18, are also anticipated by Staal. These claims further require that the method is carried out on human cells (claim 183), immune cells (claim 184) and lymphoid cells (claim 185).	Claims 183-185, depending from claim 18, are correctly characterized as requiring the method be carried out on human cells, immune cells and lymphoid cells. I do not understand the cells used by Staal et al. to be human cells, immune cells or lymphoid cells. Staal et al. use a 293.27.2 cell line transformed to express the HIV LTR fused to bacterial β-galactosidase (page 9943, second column). I do not understand 293.27.2 cells to be human cells since they have been modified to express bacterially derived elements. Further, I do not understand 293.27.2 cells to be either immune or lymphoid cells as 293.27.2 cells are a kidney cell line. Staal et al. also use a "Jurkat tri-κB" cell line. I do not understand the "Jurkat tri-

	κB" cell to be a human, immune or lymphoid cell as the generation of this cell line is not described by Staal et al. (see page 9943, second column). One of skill in the art could not determine from Staal et al. the type of cell to which "Jurkat tri-κB" refers.
Staal discloses NAC inhibition of TNF-α and stimulation in Jurkat cells (see Fig. 4, p. 9945) which are cells derived from a human T-cell leukemia cell line, and thus, are human, immune and lymphoid anticipating claims 183-185.	Staal et al. disclose the use of "Jurkat tri-κB" cells in Figure 4 (see legend). I do not understand "Jurkat tri-κB" cells to be equivalent to Jurkat cells. Figure 4 discloses the use of "Jurkat tri-κB" cells transformed to express β-galactosidase, a bacterially derived element. Further, Figure 4 does not disclose "NAC inhibition of TNF-α" as stated by the Examiner. Figure 4 rather discloses the effect of coadministration of NAC and TNF-α on β-galactosidase activity in transformed Jurkat tri-κB cells. Therefore, Staal et al. cannot teach the invention recited in claims 183-185.

Examiner Statement (Examiner Response-page(s) 113 of July 6, 2007 Final Office Action)	
Contrary to patentee's argument Staal does show that simultaneous administration of PMA, TNF-α and NAC results in "reduction of NF-κB activity in induced cells" since Staal demonstrates: -inhibition of *basal level* stimulated B-galactosidase (B-gal) cell production, > 95% inhibition of PMA stimulated cellular B-gal production, and –about 70% inhibition of TNF-α stimulated B-gal production.	Staal et al. do not "show that simultaneous administration of PMA, TNF-α and NAC results in "reduction of NF-κB activity in induced cells"." Staal et al. concurrently treats cells with NAC and the agents purported to induce NF-κB activity. Thus Staal et al. demonstrate inhibition or prevention of activation of NF-κB activity, not reducing induced NF-κB activity as recited by the claims.

107. Claim 18 and claims dependent thereon recite "a method for reducing Interleukin-1 or Tumor Necrosis Factor-α activity in mammalian cells comprising reducing NF-κB activity in the cells so as to reduce intracellular signaling caused by Interleukin-1 or Tumor Necrosis Factor-α

in the cells." (emphasis added). I understand the Examiner has alleged that Staal et al. teach administration of N-acetyl-L-cysteine to reduce intracellular signaling caused by TNF-α. I find no such teaching in Staal et al. Staal et al. disclose experiments where "NAC, PMA and TNF-α were added simultaneously" (page 9944, Figure 1 legend). The addition of NAC at the same time as PMA and TNF-α prevents activation of intracellular signaling. Staal et al. clearly state in their abstract that NAC "prevents this thiol decrease [leading to the NF-κB activation] and blocks the activation of NF-κB." Therefore, Staal et al. does not teach reduction of intracellular signaling caused by TNF-α as required by claim 18 and claims dependent thereon.

108. Further, Staal et al. clearly states that "intracellular thiol levels regulate the activation of NF-κB" (emphasis added, page 9946, second paragraph). Further, Staal et al. disclose " the mechanisms that control GSH levels also regulate those genes whose expression is dependent on the activation of NF-κB..." (emphasis added,. Page 9946, second paragraph). I understand that once NF-κB is activated by extracellular signals such as TNF-α or IL-1, modulation of intracellular thiols levels would not affect induced NF-κB activity. Thus, Staal cannot teach that administration of NAC reduces induced NF-κB activity.

109. Claim 183 is drawn to the practice of the method on "human cells." Staal et al. report the use of a 293.27.2 cell line transformed to express the "HIV LTR fused to bacterial β-galactosidase gene" and a Jurkat cell line transformed to express the "Jurkat tri-κB fusion (3κB 5.2)." (page 9943, second column). In my expert opinion, I do not understand either of these cells which express either virally-derived or bacterially derived elements to be a "human cell."

110. Further, one of skill in the art would not be able to reproduce any experiment in Staal et al. requiring the Jurkat tri-κB cells because the source of the Jurkat tri-κB fusion (3κB 5.2) is

"described elsewhere (P.S. Matilla, G.R. Crabtree, and L.A.H., personal communication)" (emphasis added, page 9943, second column). Additionally, no experimental protocol is provided as to how this construct was introduced into the Jurkat cells or whether this cell line stably or transiently expressed the construct and at what level the construct was expressed. Based on the protocols provided by Staal et al., one of skill in the art would not be able to reproduce the experiments described in Staal et al.

111. Further, Staal et al. do not perform statistical analysis on the data present in Figures 2, 3 or 4. Staal et al. note that the measurements presented in Figure 2 "were done in duplicate" (Figure 2, legend) and the data presented in Figure 4 is the "mean of duplicate samples" (Figure 4, legend). I do not understand these sample sizes to be sufficient to generate a statistical analysis and therefore, I do not understand the statistical significance of the results presented in these figures.

112. Claim 182 further limits claim 18 by requiring that the reduction of induced NF-κB activity occurs by interfering at a specific segment of the NF-κB pathway. Staal et al. state that NAC can "block NF-κB activation" (page 9947, first column) and could possibly modulate intracellular thiols to "regulate NF-κB activation at one or more points in the signal transduction cascade" (emphasis added, page 9946, second column) but does not disclose how NAC could reduce intracellular signaling caused by TNF-α as recited by the claim.

VIII. Antibiotics

A. Inherent Anticipation Rejection Based on Portions from the 1970 PDR

113. I understand the Examiner has rejected claims 6, 8, 9, 14, 18, 64-76, 80-89, 93-98, 139, 140, 144-147 and 182-185 as anticipated based on portions of the 1970 PDR, as explained by various post-filing date published references including Galdeiro et al. Microbiology (2001) 147:2697-2704; Yang et al. Nature (1998) 395:284-288; Mori et al. Eur. J. Haematol. (1997):162-170; Yasutomi et al. J. Immunology (2005) 175:8069-8076, the Baltimore '516 Patent and the undated Declaration of Dr. Manolagas cited in the July 6, 2007 Final Office Action. The cited portions of the 1970 PDR, specifically, are 1) page 1167 on Garamycin™ (gentamycin), 2) pages 1309-1310 on Sumycin™ (tetracycline), and 3) pages 1379-1380 on E-mycin™ (erythromycin). I have reviewed these portions on the 1970 PDR, the cited post-filing date documents, the Manolagas declaration and the above-identified claims. I have also reviewed the Examiner's comments in the July 6, 2007 Final Office Action regarding the above-identified documents and I disagree on several points summarized in the table below.

Examiner Statement (Rejection Summary-page(s) 105-107 of the July 6, 2007 Final Office Action)	
As detailed in Exhibit H-10 of the 90/007,503 request (herein incorporated by reference), 1970 PDR teaches administration of antibiotics such as erythromycin, gentamicin, and tetracycline to kill bacteria in animals.	The cited portions of the 1970 PDR provide dosage and administration instructions for the use of only erythromycin, gentamycin and tetracycline to treat infection.
As evidence by the Manolagas declaration, gram (-) bacteria produce lipopolysaccharides (LPS) which induce NF-κB regulated cytokine production. Antibiotics that kill gram-negative bacteria thereby act to inherently reduce LPS-induced and NF-κB regulated cytokine production.	Antibiotics have no effect on NF-κB activity. Killing bacteria does not reduce the amount of LPS already present. LPS induces activation of NF-κB. Removing LPS (which is not an effect of an antibiotic) merely prevents further induction of NF-κB, but cannot reduce induced NF-κB activity. Furthermore, certain species of gram-negative bacteria, such as *Brucella*, produce LPS which does not induce production of cytokines (as discussed

	below in paragraph 123).
The instant claims are drawn to reducing NF-κB activity in eukaryotic (e.g. claims 1, 2 or 5) or mammalian cells (e.g. claim 26) to effect inhibited expression of a gene under transcription control of NF-κB.	Claims 1, 2, 5 and 26 are no longer under review.
For example, NF-κB activity can be effected by diminishing induced NF-κB mediated intracellular signaling (claims 6-9) or decreasing the level of NF-κB not bound in an NF-κB –I-κB complex (claim 20) to inhibit associated gene expression of a cytokine protein (claims 5 and 18) and reduce the effects of bacterial lipopolysaccharides (claim 12-17) on eukaryotic (e.g. mammalian) cells.	Claims 5, 7, 1-13 and 15-17 are not under review. The claims under review recite what they recite. Claim 6 and claims dependent thereon are correctly characterized as requiring diminishing induced NF-κB activity. Claims 14, 18 and claims dependent thereon are incorrectly characterized. Claim 14 and claim dependent thereon require "reducing bacterial lipopolysaccharide-induced expression of cytokines in mammalian cells." Claim 18 requires reducing intracellular signaling caused by Interleukin-1 or Tumor Necrosis Factor-α in mammalian cells.
The instant claims are drawn to a method of inhibiting NF-κB activity (e.g. diminishing bacterially LPS induced NF-κB expression of cytokines mediated, for example, through intracellular signaling: in claims 6, 8-10) and associated gene (claim 1-2) expression (e.g. of a cytokine protein: claim 5) in a eukaryotic cell.	The claims under review require reducing induced NF-κB activity.
The 1970 PDR teaches the administration of antibiotics (erythromycin, gentamycin, tetracycline) to treat gram-negative bacterial infections.	The 1970 PDR provides instruction for the use of the antibiotics (erythromycin, gentamycin, tetracycline) to treat specific species of both gram positive and gram negative bacteria. Only some gram negative bacteria produce LPS.
Erythromycin, for example, is active against *H. pertusis* infections (Id. At 1379); and gentamicin sulfate (trade name GARAMYCIN) treats infections caused by *Pseudomona aeruginosa, Aerobacter aerogenes, E.coli, Proteus vulgaris,* and *Klebsiella pneumoniae* (id at 1167).	Erythromycin is only sometimes active against the infections listed. First, the 1970 PDR page 1379 clearly states that erythromycin has "a lower order of activity against the gram-negative" bacteria than against gram-positive bacteria.
Tetracycline is a broad spectrum antibiotic (trade name SUMYCIN) which treats a variety of infectious gram (-) bacteria, including *E coli* and *Shigella* (id at 1309).	Tetracycline is only sometimes active against the infections listed. The 1970 PDR further discloses at page 1309, "Microorganisms that have become insensitive to one tetracycline invariably

	exhibit cross-resistance to other tetracyclines." Further, on page 1310, the 1970 PDR states "A few strains of pneumococci, *E. coli* and shigellae also have been reported as resistant."
Gram-negative bacteria produce lipopolysaccharide (LPS), which when in contact with human cells induces NF-κB activity resulting in the production of cytokines regulated by NF-κB.	This is not an accurate statement. As discussed below, certain species of gram-negative bacteria, such as *Brucella*, produce LPS which does not induce production of cytokines (as discussed below in paragraph 123).
Cytokines whose genes are regulated by NF-κB (at least in part) include tumor necrosis factor alpha (TNF-α) and interleukins 2, 6, 8 and 10 (IL 2, 6, 8 and 10). Eg Galdiero; Yang; and Mori. As noted in Galdiero (page 2700), "The lowest concentration of LPS able to induce cytokine release was 10 ng ml^{-1}". Once produced by gram-negative bacteria, LPS activates NF-κB, which translocates to the nucleus and binds to the NF-κB recognition sites on genes that are regulated, at least in part, by NF-κB. Eg. Baltimore patent, col. 2, lines 54-57 ("release of active NF-κB from the IκB - NF-κB complex has been shown to result from stimulation of cells by a variety of agents, such as bacterial lipopolysaccharide...").	These statements all presume that antibiotics reduce induced NF-κB activity. As explained, antibiotics cannot reduce induced NF-κB activity.
In treating gram-negative bacterial infections, antibiotics kill bacteria or impair bacterial function thereby reducing bacterial production of LPS (Manolagas Declaration, ¶¶33-40).	This is a misleading statement. Antibiotics sometimes kill bacteria, sometimes impair bacterial function and sometimes have no effect on bacteria which are resistant. Plus, antibiotics sometimes reduce bacterial production of LPS, sometimes have no effect on LPS production, and sometimes result in an increase in circulating levels of LPS in the body. See, eg. paragraphs 128-130 of my first Declaration and paragraphs 113 and 122-123 below.
For example, erythromycin has been shown to inhibit the activation of NF-κB in T cells; and in normal and inflamed human bronchial epithelial cells along with modulating IL-8 expression. See Yasutomi et al. at page 8068 at the bottom of the right column and footnotes 5-7 (citations	The inhibition of "activation of NF-κB" by erythromycin is a method different from reducing induced NF-κB activity as required by the claims under review.

therein).	
Yasutomi further demonstrated the ability of tetracycline to inhibit lipopolysaccharides and NF-κB activation in human monocyte-derived dendritic cells and suppress NF-κB induced cytokine (e.g. IL-6, 10, 12p70, 13 and IFN-γ) production. See abstract, page 8071-8073, see Table 1 and Figures especially 1-3 and 8.	Yasutomi et al. do not describe any experiment using tetracycline. In any event, the administration of the antibiotic erythromycin prior to stimulation, which is disclosed by Yasutomi et al. (page 8070, first column), is a method different from reducing induced NF-κB activity. Therefore, Yasutomi et al. do not describe that antibiotics reduce induced NF-κB activity.
Thus, by blocking LPS that reaches the cell, administration of the 1970 PDR antibiotics to combat gram-negative bacterial infections would necessarily reduce NF-κB activity in human cells and thereby reduce cytokine expression.	Yasutomi et al. do not disclose any evidence describing "blocking LPS" by erythromycin or any antibiotic, nor does the July 6, 2007 Final Office Action point to where this is described in Yasutomi et al. or any other reference.
As such, as shown on an element-by-element claim basis in Exhibit H-10 of the 90/007,503 request (incorporated by reference), the disclosed 1970 PDR antibiotics would inherently anticipate the subject claims requiring LPS-induced NF-κB activity.	I have read the 1970 PDR and Exhibit H-10 and I do not find them to teach the method of the claims under review. The 1970 PDR acknowledged antibiotic resistance on pages 1309 and 1310 of the 1970 PDR. More importantly, antibiotics cannot reduce LPS-induced NF-κB activity as recited in the claims under review.

114. Antibiotics cannot reduce induced NF-κB activity. Antibiotics, according to the Examiner's theory, sometimes kill bacteria, thereby allowing LPS to be cleared from a subject. In the case where LPS has already induced NF-κB activity, removal of LPS does not change such induced activity. The absence of LPS does not reduce induced NF-κB activity. LPS, when present, induces NF-κB activity, but when absent does not. Thus, according to the Examiner's theory, antibiotics can only prevent induction of NF-κB by LPS but under no theory or evidence presented in the July 6, 2007 Final Office Action can antibiotics reduce induced NF-κB activity. Further, in Exhibit H-10, referred to in the July 6, 2007 Final Office Action, the same statement appears next to claims each of 6, 8, 9, 14 and 18 regarding the 1970 PDR: "Teaches

administration of antibiotic for treatment of gram negative bacterial infection." This is not an accurate statement in that the 1970 PDR provides instruction for the use of antibiotics to treat both gram positive and gram negative bacterial infections. Notably, on page 1379, third column, the 1970 PDR discloses that erythromycin has a "lower order of activity against gram-negative" bacteria than against gram positive bacteria. Therefore, I do not understand the 1970 PDR to provide instruction for the use of the antibiotics solely for resolving gram negative bacterial infections. According to the express teaching of the 1970 PDR, the antibiotics are only sometimes effective.

115. Claim 6 and claims dependent thereon recite a "method for diminishing induced NF-κB-mediated intracellular signaling" (emphasis added). I understand the Examiner has alleged that the 1970 PDR teaches administration of antibiotics to kill gram-negative bacteria which produce LPS thereby reducing NF-κB activity. I find no such teaching in the 1970 PDR. The 1970 PDR provides dosage and administration instructions for the use of erythromycin, gentamycin and tetracycline. There antibiotics have no direct effect on NF-κB activity. Killing bacteria does not reduce the amount of LPS already present. Though LPS induces activation of NF-κB, removing LPS (which is not a direct effect of an antibiotic) merely prevents further induction of NF-κB. Thus the 1970 PDR provides no evidence that antibiotics can diminish induced NF-κB-mediated intracellular signaling as recited by claim 6.

116. Claim 8 and claims dependent thereon recite a "method for modifying the effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling." The 1970 PDR does not demonstrate that administration of antibiotics modifies the effects of external influences which induce NF-κB-mediated intracellular signaling.

As I have discussed in paragraph 12, the 1970 PDR provides dosage and administration instructions. Thus, the 1970 PDR cannot teach a method for modifying effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling as recited by claim 9.

117. Claim 9 and claims dependent thereon recite a method for "reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." The 1970 PDR does not teach any method which necessarily results in an antibiotic-mediated reduction in the level of expression of any gene which is activated by extracellular influences which induce NF-κB-mediated intracellular signaling.

118. Further, the 1970 PDR does not demonstrate that administration of antibiotics reduced the level of expression of an activated gene. Again, the 1970 PDR provides dosage and administration instructions for the use of erythromycin, gentamycin and tetracycline. These antibiotics sometimes kill bacteria thereby allowing the body of a subject to clear LPS. The absence of LPS does not reduce the level of expression of a gene activated by NF-κB. Further, the 1970 PDR does not disclose any gene that is activated by NF-κB. Thus, the 1970 PDR does not teach the reducing, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated signaling.

119. Claim 14 and claims dependent thereon recite a method for "reducing bacterial lipopolysaccharide-induced expression of cytokines in mammalian cells, which method comprises reducing NF-κB activity in the cells so as to reduce bacterial lipopolysaccharide-induced expression of said cytokines in the cells." The Examiner has alleged that the

administration of the antibiotics erythromycin, gentamycin and tetracycline to treat gram negative bacterial infections necessarily practices the method of claim 14. I respectfully disagree. The 1970 PDR provides dosage and administration instructions for the use of these three antibiotics. These antibiotics have no effect on NF-κB activity. Killing bacteria does not reduce the amount of LPS already present. Removing LPS (which is not an effect of an antibiotic) merely prevents further induction of NF-κB, but cannot reduce induced NF-κB activity. Therefore, the 1970 PDR cannot teach a method for reducing bacterial lipopolysaccharide-induced expression of cytokines in mammalian cells, which method comprises reducing NF-κB activity in the cells so as to reduce bacterial lipopolysaccharide-induced expression of said cytokines in the cells.

120. Claim 18 and claims dependent thereon recite a method for "reducing Interleukin-1 or Tumor Necrosis Factor-α activity in mammalian cells comprising reducing NF-κB activity in the cells so as to reduced intracellular signaling caused by Interleukin-1 or Tumor Necrosis Factor-α in the cells." The Examiner has alleged that the administration of the antibiotics erythromycin, gentamycin and tetracycline to treat gram negative bacterial infections inherently teaches the method of claim 18. I find no such teaching in the 1970 PDR. The 1970 PDR provides dosage and administration instructions for the use of these three antibiotics. These antibiotics have no effect on NF-κB activity and no effect on TNF-α. Killing bacteria, under the theory of the July 6, 2007 Final Office Action, reduces the amount of LPS. It is unclear how removing LPS reduces signaling caused by TNF-α or IL-1. Therefore, the 1970 PDR cannot teach a method for reducing intracellular signaling caused by Interleukin-1 or Tumor Necrosis Factor-α in the cells.

121. Claims 64, 65 and 69 further limit claim 6, claims 75, 76 and 80 further limit claim 8, claims 88, 89 and 93 further limit claim 9, claim 139-140 and 144 further limit claim 14 and claims 177-178 and 182 further limit claim 18 by requiring that the reduction of induced NF-κB activity occurs by interfering at a specific segment of the NF-κB pathway. The Examiner has alleged that the administration of the antibiotics erythromycin, gentamycin and tetracycline to treat gram negative bacterial infections necessarily practices the method of claims dependent on claims 6, 8, 9, 14 and 18, as listed above. I respectfully disagree. The 1970 PDR does not disclose any mechanism by which these three antibiotics reduce induced NF-κB. In Exhibit H-10, as relied on by the July 6, 2007 Final Office Action, I note the same statement listed next to claims 64-65, 69, 75-76, 80, 88-89, 93, 139-140,144, 177-178 and 182: "Inherent. Reduction in LPS due to administration of antibiotic necessarily decreases NF-κB activation.." (emphasis added). I find no such teaching in the 1970 PDR. The 1970 PDR provides dosage and administration instructions for the use of these three antibiotics. Further, the statement cited from Exhibit H-10 clearly states that NF-κB activation is decreased thus reaffirming that these three antibiotics cannot reduce induced NF-κB activity.

122. In addition to the points I raised in my previous declaration, at ¶¶127-131, I would like to elaborate on the phenomenon of antibiotic resistance. The Examiner alleged, referring to Exhibit H-10, that reducing NF-κB is "Inherent. Reducing LPS by administration of antibiotic for treatment of gram negative bacterial infection necessarily reduces NF-κB activity." I respectfully disagree. The instances of antibiotic resistance among the species of bacteria cited in the July 6, 2007 Final Office Action and listed in the 1970 PDR as treatable with gentamycin, tetracycline and erythromycin are summarized in Table 1, a copy of which is attached as **Exhibit 15**. Antibiotic resistance among bacteria is not a trivial matter. For example, *Shigella*, which the

Examiner lists as treatable with tetracycline, has long been known to be resistant to tetracycline. Roberts (2003), (a copy of which is attached as **Exhibit 17**) states that "the first tetracycline-resistant bacterium, *Shigella dysenteriae*, was isolated in 1953, and the first multi-drug resistant *Shigella* pathogen was isolated in 1955....By 1960, mutli-drug resistant *Shigella* species represented almost 10% of the strains tested in Japan, whereas a more recent study showed that ≥60% of the isolates of *Shigella flexneri* recovered were resistant to tetracycline, streptomycin, and chloramphenicol.." (emphasis added, page 464, first column). Clearly, the incidence of antibiotic resistance as described in Roberts and summarized in the submitted Table 1 illustrates that the administration of antibiotics does not consistently result in killing of gram-negative bacteria thereby reducing LPS.

123. Further, the Examiner alleged, referring to Exhibit H-10, page 11 that "Reducing LPS by administration of antibiotic for treatment of gram negative bacterial infection necessarily modifies effects of external influences on a eukaryotic cell, which external influences induce NF-κBB-mediated intracellular signaling" and on page 14 "necessarily reduces, in eukaryotic cells, the level of expression of genes which are activated by extracellular influences which induce NF-κB-mediated intracellular signaling." Further, the Manolagas Declaration states that "when LPS comes into contact with cells in the human body, it is now known to induce NF-κB activity and, in turn, increase the production of cytokines that are regulated by NF-κB activity." (see page 17, paragraph 34). I respectfully disagree with the above statements. Intracellular gram-negative pathogens, such as *Brucella*, produce LPS, but do not induce an inflammatory response. Lapaque et al. (2006), a copy of which is attached as **Exhibit 18**, disclose that *Brucella*, and other species of bacteria such as *Legionella pneumophila*, produce a non-classical LPS molecule which does not stimulate the inflammatory response. Lapaque et al. state "some Gram-negative

bacteria that replicate extensively to reach high numbers in tissues and cells do not induce endotoxic shock. For instance, *Brucella* and other intracellular pathogens specialized in chronic disease outcome and not acute disease outcome such as salmonellosis must hide into host cells and lower the host responses in order to survive and replicate" (page 408). Further, Lapaque et al. state that "the low endotoxicity of LPS carrying the non-classical lipid A structures used in this study, and show that these failed to induce high levels of TNF-α even at high concentrations" (page 409). Likewise, Dixon and Darveau (2005), a copy of which is attached as **Exhibit 18**, describe heterogeneity in LPS molecules produced by non-enteric gram-negative bacteria. Dixon and Darveau state that "early studies examining endotoxin lethality in mice demonstrated that LPS obtained from Gram-negative oral anaerobic bacteria were significantly reduced in potency when directly compared with enterobacterial LPS" (page 587). Therefore, contrary to the Examiner's assertions in Exhibit H-10 and the statements in the Manolagas declaration, not all gram-negative bacteria produce an LPS molecule capable of stimulating the inflammatory response thus administration of antibiotics to resolve gram-negative infections does not necessarily result in modulation of the inflammatory response. Thus, even if I accept all the cited prior and post art, and I do not for reasons discussed herein and in my previous Declaration, no evidence is provided demonstrating that antibiotics reduce induced NF-κB activity.

124. I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent.

Inder Verma, Ph.D.

Oct 19/2007
Date

EXHIBIT D

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo

JOURNAL OF VIROLOGY, Aug. 1990, p. 4037–4041
0022-538X/90/084037-05$02.00/0

Vol. 64, No. 8

Inducible Nuclear Factor Binding to the κB Elements of the Human Immunodeficiency Virus Enhancer in T Cells Can Be Blocked by Cyclosporin A in a Signal-Dependent Manner

ALBRECHT SCHMIDT,[1] LOTHAR HENNIGHAUSEN,[2] AND ULRICH SIEBENLIST[1]*

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases,[1] and Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases,[2] Bethesda, Maryland 20892

Received 26 February 1990/Accepted 4 May 1990

Cyclosporin A (CsA) is thought to exert its immunosuppressive effects by inhibiting the expression of a distinct set of lymphokine genes which are induced upon T-cell activation, among them the gene coding for interleukin-2. In addition, the activation of the human immunodeficiency virus (HIV) is partially suppressed. To better understand the molecular mechanisms underlying suppression by CsA, we have investigated the effects of this drug on transcription factors in T cells. Here we report that the formation of two distinct mitogen-inducible DNA-binding complexes, the κB complex within the HIV enhancer and the NFAT-1 complex within the interleukin-2 enhancer, is inhibited in the presence of CsA. The κB-binding activity with the HIV enhancer is inhibited only if it is activated via the mitogen phytohemagglutinin, whereas phorbol myristate acetate-mediated activation is completely insensitive to the drug. This suggests a model in which functionally indistinguishable κB complexes can be activated via two separate pathways of signal transduction distinguishable by CsA.

Cyclosporin A (CsA) is a clinically important immunosuppressive drug which suppresses induction of a number of genes, including that coding for interleukin-2 (IL-2), in T cells (9, 25), and to some extent expression of human immunodeficiency virus (HIV) (26, 28). The molecular mechanisms of this suppression are unknown, but it has been shown that CsA inhibits the induced expression of several genes at the transcriptional level (10, 14). Two tandemly repeated κB sequence elements are essential to the activation of the HIV genome, and these elements confer enhancer function (19). This enhancer element binds inducible nuclear factors in T cells, the NF-κB factor, and additional, possibly related factors including HIVEN-86 (2, 3, 8, 19). κB nuclear factor-binding elements also play important roles in the activation of the gene coding for IL-2 and other genes in T cells (11, 15, 16, 22). In addition to κB, other IL-2 enhancer elements appear to mediate signal transduction, and some of these elements show inducible factor binding after activation of Jurkat T cells (4, 6, 11, 16, 22, 24). Among the elements that show inducible factor binding is the site termed IL-2-IIA by us (4) and NFAT-1-binding site by others (24), which has been implicated directly in playing a major role during transcriptional activation of IL-2 (6, 24). We show here that the activation of the κB and NFAT-1 complexes in T cells is sensitive to CsA, whereas activation of an inducible AP-1 complex is not. This suggests selective action of the drug. We further show that the HIV-κB-binding complex as well as the function of the HIV enhancer in transfection experiments is completely inhibited when mediated through a phytohemagglutinin (PHA)-induced signal but is insensitive to a phorbol myristate acetate (PMA)-induced signal.

We investigated the effect of CsA on the activation of the κB-binding complex of the HIV long terminal repeat in T cells with the electrophoretic mobility shift assay. A radioactively labeled oligonucleotide fragment representing the κB-binding site (nucleotides −104 to −79, 5′-GGGACTTT CCGCTGGGGACTTTCCAG-3′ [19]) was incubated with nuclear extracts (10 μg of protein) in a total volume of 20 μl {40 mM NaCl, 40 mM KCl, 0.2 mM EDTA, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], approximately 7% glycerol [vol/vol], 0.5 mM dithiothreitol, 25 μM NPGB, 1 mM phenylmethylsulfonyl fluoride, 2 μg of poly[dI-dC)] [Pharmacia]} for 30 min at room temperature. Resulting complexes were separated on a 4% low-ionic-strength polyacrylamide gel (27) in a cold room. Activation of T cells by PHA-PMA induced the binding of a major complex (termed B) to the κB enhancer elements (Fig. 1, lane 3). An additional inducible complex (termed A), closely spaced and slightly more slowly migrating, could also be discriminated (Fig. 1, lane 3). Although detected repeatedly, the A shift was weak and variable in strength; it was most clearly seen when cells were stimulated with PHA alone (Fig. 1, lane 5) but never seen when cells were stimulated with PMA alone (Fig. 1, lane 7). The nature of the two constitutively present complexes is unknown; they were essentially unaffected by the activation protocols. The major inducible shift (B complex) appears identical to the previously reported (2, 3, 19) inducible shift obtained with similar HIV κB oligonucleotide probes because (i) the shift was not detected with a mutant enhancer, nor was the B shift competed for by this mutant (data not shown; the mutation κB/M [19] is 5′-CTCACTTTCCGCTGCTCACTTTCCAG-3′), and (ii) this induced shift comigrated with a shift formed with nuclear extracts from Namalwa cells, which contain a constitutively activated κB complex (13) (data not shown). The activable binding activity giving rise to this shift was ascribed to the nuclear factor NF-κB and possibly other closely related factors (2, 3, 8, 19). Addition of CsA during stimulation with both PHA and PMA significantly reduced the major shift (B shift) and completely abrogated the A shift (Fig. 1, lane 4).

Since the induction of the major κB shift was only partially

* Corresponding author.

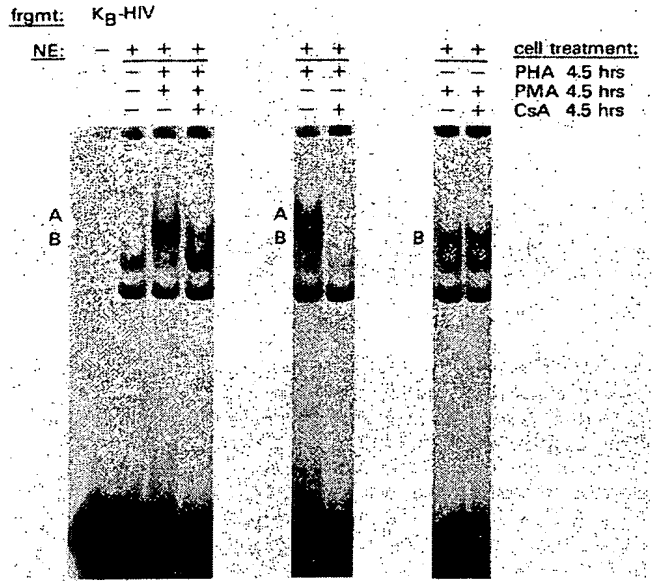


FIG. 1. Dependence of CsA inhibition of HIV-κB binding activity on the inducing signal. Complementary single-stranded oligonucleotide fragments representing the HIV enhancer (frgmt: κB-HIV; nucleotides −104 to −79, with directly abutting 3′-end *Sst*I overhangs) were radioactively labeled with polynucleotide kinase, annealed, and then incubated with nuclear extracts. Nuclear extracts (NE) were prepared from untreated (−) Jurkat cells or cells treated for 4.5 h with various agents (PHA, 1 μg/ml; PMA, 20 ng/ml; CsA, 1 μg/ml) (+) by hypotonic swelling of cells, Dounce homogenization, salt extraction of the resulting nuclei, and precipitation of the extracted nuclear proteins with ammonium sulfate, as described by Shapiro et al. (23). Lane −, Free labeled oligonucleotides without nuclear extracts added; lanes +, nuclear extracts added. B and A, Positions of shifts B and A, respectively. In addition, two constitutively present complexes are seen.

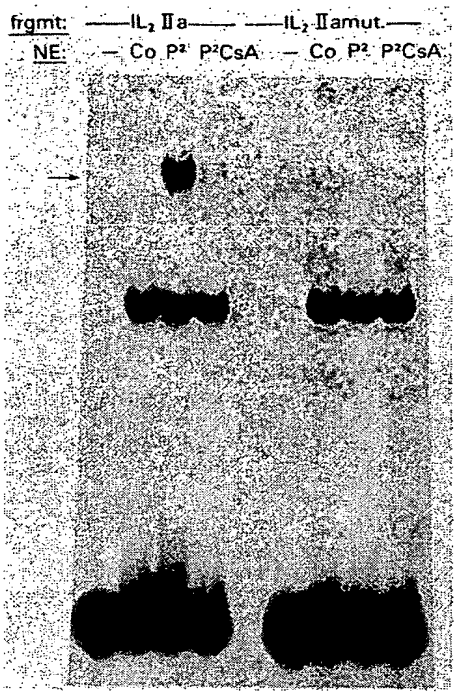


FIG. 2. Sensitivity of inducible NFAT-1 nuclear factor complex formed with IL-2 promoter region IIA to CsA. Double-stranded oligonucleotide fragments (frgmt) representing the NFAT-1-binding site IL-2-IIa (4, 24) (IL-2 nucleotides −288 to −266; 5′-AAGGAG GAAAAACTGTTTCATAC-3′ [left]) or a mutant of this binding site (IL-2-IIamut; AAGGAGGCAAAACTGTTGCATAC [right]) (both sets of oligonucleotides have directly abutting 5′ *Bgl*II [upper strand] or *Bam*HI [lower strand] overhangs) were radioactively labeled by filling in the ends with Klenow enzyme. The oligonucleotides were then incubated with nuclear extracts, and the resulting complexes were separated as described in the legend to Fig. 1. Nuclear extracts (NE) were prepared from Jurkat cells treated with various agents, as described in the legend to Fig. 1. Lanes Co, Untreated cells; lanes P², PHA-plus-PMA treatment; lanes P²CsA, PHA plus PMA plus CsA; lanes −, free labeled oligonucleotides without nuclear extracts added. →, Position of the inducible shift. The additional nonspecific shift was largely unaffected by the various types of cell treatment, and the nature of the shift is unknown.

suppressed when the broadly acting, mitogenic stimuli PHA and PMA were used, we investigated the activity of CsA with each agent alone. PHA mimics induction via the T-cell receptor, while PMA stimulation is thought to be mediated at least in part via direct activation of protein kinase C. While PHA-mediated induction of complexes binding to the κB enhancer was completely abrogated by CsA (Fig. 1, lane 6; no B or A shifts), the PMA-induced shift was not at all affected (Fig. 1, lane 8). These data were confirmed with multiple different independent extracts. We conclude that very similar or identical κB complexes were activated via different pathways during stimulation with PHA versus PMA and that these pathways were distinguishable by their sensitivities to CsA.

As shown by electrophoretic mobility shift assay, an oligonucleotide encompassing the IL-2 enhancer site IIA (NFAT-1-binding site) bound a complex inducible upon activation of Jurkat T cells with PHA and PMA (Fig. 2, left panel, arrow). However, the addition of CsA during the cellular activation phase completely abolished this binding. When a mutant of this element was used, no inducible binding activity could be detected in any of the variously treated cellular extracts (Fig. 2, right panel). This shows the sequence specificity of the induced factor, as do previous data (6). In addition to the findings reported here, we have observed good activation of NFAT-1 with PHA alone but not with PMA alone (data not shown). Therefore, the complete suppression of NFAT-1 in the presence of both PHA and PMA is consistent with the notion that CsA interferes with some necessary action(s) initiated by PHA but not with those initiated by PMA (10). Direct addition of CsA to a prepared nuclear extract from activated cells had no effect on the factor binding, including binding of κB-binding factors (data not shown), which suggests that inhibition occurs during the activation phase of NFAT-1 or the κB complex.

The AP-1 site is known to confer inducibility on many genes. To test whether the inducible binding to this element (18) is sensitive to CsA as well, we performed electrophoretic mobility shift assays with the identical CsA-treated and untreated nuclear extracts described above. Inducible binding activity was completely insensitive to CsA (Fig. 3), even when PHA was used alone as a stimulus. This experiment shows that some activable binding complexes are not inhibited by CsA, and therefore AP-1 represents a control for the inhibition of the NFAT-1 and κB factors. This experiment

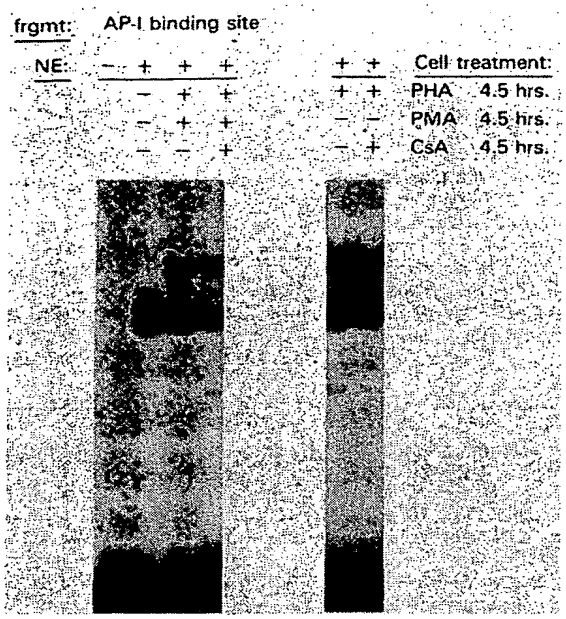


FIG. 3. Inducible binding to an AP-1 site is insensitive to CsA. The double-stranded oligonucleotide fragment (frgmt) bearing an AP-1 consensus core sequence (5'-AGAAATAGATGAGTCAAC AG-3', with CG dinucleotide 5' overhangs on both strands [21]) was radioactively labeled by filling in the ends with Klenow enzyme and was subjected to electrophoretic mobility shift assay with nuclear extracts (NE) from cells treated (+) or not treated (−) as indicated and as described in the legend to Fig. 1. The inducible band (upper band) is essentially insensitive to CsA. The nature of the lower constitutively present band shift is unknown. Lane −, Free labeled oligonucleotides without nuclear extracts added; lanes +, nuclear extracts added.

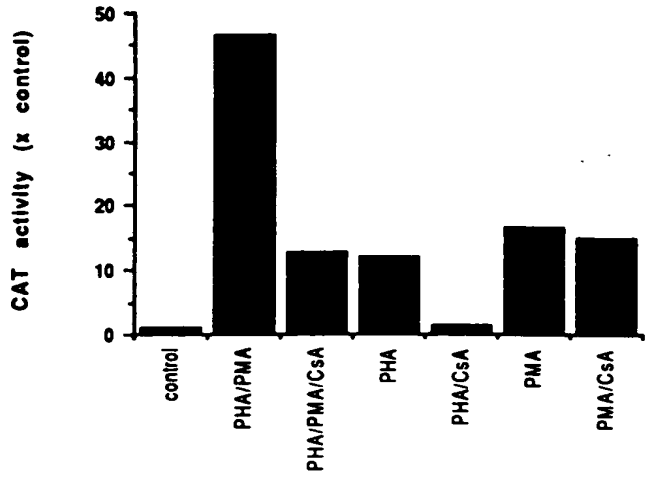


FIG. 4. Effects of CsA upon CAT inducibility conferred by HIV enhancer (κB-binding sites) in Jurkat cells. CAT activities determined after the transfection with a construct in which the HIV κB enhancer is driving the expression of the CAT gene are shown (κB-WAP-CAT). To make this construct, a 112-base-pair fragment from the mouse WAP promoter, extending from −88 to +24 (*Xba*I to *Kpn*I) (5), was cloned into a CAT vector containing no promoter or enhancer sequences (pA10 CAT 3M), resulting in the minimal promoter construct WAP-CAT. Two copies of the wild-type κB-binding region (nucleotides −104 to −79) were integrated in the same orientation into an *Sst*I site at −88 of the WAP-CAT construct via *Sst*I 3' overhangs, creating κB-WAP-CAT. We also integrated two copies of the mutant κB/M (see above) to create κB/M-WAP-CAT. The activities under various types of cell treatment (indicated below each bar) are expressed relative to the total CAT activity found with untreated, transfected cells, which was arbitrarily given a value of 1.0. Results of a single representative experiment are shown. In repeated experiments with the same construct, qualitatively identical results were obtained.

also suggests that only some activation paths initiated by PHA are sensitive to CsA in T cells (10).

To correlate the signal-dependent changes in factor binding in vitro with transcriptional regulatory function in vivo, HIV wild-type or mutant enhancer-driven chloramphenicol acetyltransferase (CAT) constructs containing a minimal whey acidic protein (WAP) promoter were transfected into Jurkat cells and tested for CAT activity after various cellular stimulations (Fig. 4). Several independent transient transfections of the constructs were carried out by using a modified DEAE method in which cells are slightly swollen with hypotonic medium before transfection (17). Following a 36- to 48-h incubation period after transfection, cells were split for the individual treatments, eliminating the need for internal transfection efficiency controls. These cells were then treated for 4.5 h with PHA, PMA, and CsA, and CAT assays were performed as previously described (20). Regulation of transcription conferred by the HIV enhancer correlated directly with the mobility shift binding studies of the κB complex. Specifically, CsA inhibited the PHA-derived activation signal but not the PMA signal. Thus, CsA caused no general inhibition of cellular function. The activation mediated by PHA and PMA exceeded that of either agent alone, and as expected, costimulation with PHA and PMA was only partially inhibited by CsA. The transfection experiments were also done with a 10-fold-lower level of CsA (0.1 μg/ml), and we observed qualitatively identical results (data not shown). In additional controls, the HIV enhancer mutation κB/M, which prevented binding of κB-like complexes (19), also completely abrogated the inducibility of CAT activity by PHA or PMA (or both), as did a construct with the minimal WAP promoter only (WAP-CAT) (data not shown). Our transfection data are consistent also with earlier transfection reports which demonstrated that parts or all of the HIV long terminal repeat confers inducibility by signals originating from the antigen receptor or originating with PMA-mediated activation but that only the signal from the antigen receptor is sensitive to CsA (26, 28).

CsA suppressed the induction-dependent binding activity in T cells of two apparently unrelated DNA-binding complexes, the NF-κB-like complex formed with the HIV enhancer and the NFAT-1 complex formed with the IL-2-IIA site. Additional inducible DNA-binding activities may be affected, which would be consistent with a broad action of this drug as documented by the relatively large number of genes in T cells which are suppressible (10, 29). On the other hand, not all inducible complexes were suppressed, as exemplified by the induction of the AP-1 complex in the presence of the drug. Surprisingly, suppression of the κB complex of the HIV enhancer in T cells occurred only when the cells were activated by PHA (which is likely to act through the antigen receptor) but not when activated by PMA. Apparently, identical κB complexes were formed after the delivery of either signal, but the paths for transducing the signal must have been different. When PHA plus PMA was used to activate cells, inhibition of the κB complex was only partial, which suggests that the PMA-initiated

signals remained unaffected by CsA. Since the NFAT-1 factor required stimulation by PHA as opposed to PMA, it was very sensitive to CsA even in the presence of both PHA and PMA.

After completion of this work, a related report appeared which demonstrated CsA inhibition of the NFAT-1- and the κB-binding activities in PHA-PMA-stimulated T cells (7). The partial inhibition of the κB-binding activity in the presence of CsA after induction with these combined signals was not analyzed further, however.

The results of the transfection experiments with HIV enhancer-driven CAT constructs demonstrated that the in vivo effects closely corresponded to the results of κB-binding studies in vitro. The PHA-stimulated but not the PMA-stimulated cells were sensitive to CsA. Thus, CsA appears to inhibit a step(s) in cellular activation which follows PHA or antigen receptor stimulation but does not inhibit the PMA signal. Since it is known that stimulation via the antigen receptor leads to production of inositol phosphate derivatives and diacylglycerol even in the presence of CsA (12), the suppression must occur downstream of these second messengers or involve an as yet unidentified second messenger. Also, activation of AP-1 with PHA was not affected by CsA, which suggests that only some PHA-derived signals are suppressible.

It is not known what links the activation of the κB and NFAT-1 factors. NFAT-1 appears to involve the synthesis of an induced gene, since cycloheximide interferes with NFAT-1 activation (4, 24). On the other hand, the κB factor(s) is already present in the cell and requires only release from a cytoplasmic inhibitor (1). Considering that several similar but possibly distinct nuclear factors may bind to the HIV κB region, we cannot rule out that such factors are separately activated depending on the inducing signal. This could be the cause of the observed differential sensitivity to CsA. Whether the same or a distinct factor(s) binds as a result of the two activation signals, these signal-transducing paths converge functionally at the DNA level, as shown by the transfection experiments. It is possible that different kinases (or other modifications) effect the release of NF-κB from its inhibitor (1) and that some of these kinases in turn are regulated by distinct signaling pathways.

We thank A. S. Fauci and K. Kelly for support and encouragement and critical reading of the manuscript, D. Levens for the AP-1 oligonucleotide, and M. Brunvand for IL-2 oligonucleotides and initial help with nuclear extraction.

A.S. was supported by the Stiftung Volkswagenwerk.

LITERATURE CITED

1. **Baeuerle, P. A., and D. Baltimore.** 1989. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science **242**: 540–545.
2. **Böhnlein, E., J. W. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. Greene.** 1988. The same inducible nuclear protein regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. Cell **53**:827–836.
3. **Böhnlein, E., M. Siekevitz, D. W. Ballard, J. W. Lowenthal, L. Rimsky, H. Bogerd, J. Hoffman, Y. Wano, B. R. Franza, and W. C. Greene.** 1989. Stimulation of the human immunodeficiency virus type 1 enhancer by the human T-cell leukemia virus type I *tax* gene product involves the action of inducible cellular proteins. J. Virol. **63**:1578–1586.
4. **Brunvand, M. W., A. Schmidt, and U. Siebenlist.** 1989. Nuclear factors interacting with the mitogen-responsive regulatory region of the interleukin-2 gene. J. Biol. Chem. **263**:18904–18910.
5. **Campbell, S. M., J. M. Rosen, L. G. Hennighausen, U. Strech-Jurk, and A. E. Sippel.** 1984. Comparison of the whey acidic protein genes of the rat and mouse. Nucleic Acids Res. **12**: 8685–8697.
6. **Durand, D. B., J.-P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree.** 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8**:1715–1724.
7. **Emmel, E. A., C. L. Verweij, D. B. Durand, K. M. Higgins, E. Lacy, and G. R. Crabtree.** 1989. Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. Science **246**:1617–1620.
8. **Franza, B. R., S. F. Josephs, M. Z. Gilman, W. Ryan, and B. Clarkson.** 1987. Characterization of cellular proteins recognizing the HIV enhancer using a microscale DNA-affinity precipitation assay. Nature (London) **330**:391–395.
9. **Granelli-Piperno, A., L. Andrus, and R. M. Steinman.** 1986. Lymphokine and nonlymphokine mRNA levels in stimulated human T cells: kinetics, mitogen requirements, and effects of cyclosporin A. J. Exp. Med. **163**:922–937.
10. **Gunter, K. C., S. G. Irving, P. F. Zipfel, U. Siebenlist, and K. Kelly.** 1989. Cyclosporin A-mediated inhibition of mitogen-induced gene transcription is specific for the mitogenic stimulus and cell type. J. Immunol. **142**:3286–3291.
11. **Hoyos, B., D. W. Ballard, E. Böhnlein, M. Siekevitz, and W. C. Greene.** 1989. Kappa B-specific DNA binding proteins: role in the regulation of human interleukine-2 gene expression. Science **244**:457–460.
12. **Imboden, J. B., and A. Weiss.** 1988. The initiation of human T lymphocyte activation. Prog. Allerg. **42**:246–279.
13. **Kawakami, K., C. Scheidereit, and R. G. Roeder.** 1988. Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc. Natl. Acad. Sci. USA **85**:4700–4704.
14. **Kronke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldmann, and W. C. Greene.** 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. Proc. Natl. Acad. Sci. USA **81**:5214–5218.
15. **Lenardo, M. J., C. M. Fan, T. Maniatis, and D. Baltimore.** 1989. The involvement of NF-KB in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell **57**:287–294.
16. **Lenardo, M. J., A. Kuang, A. Gifford, and D. Baltimore.** 1988. NF-KB protein purification from bovine spleen: nucleotide stimulation and binding site specificity. Proc. Natl. Acad. Sci. USA **85**:8825–8829.
17. **Lieber, M. R., J. E. Hesse, K. Mizuuchi, and M. Gellert.** 1987. Developmental stage specificity of the lymphoid V(D)J recombination activity. Genes Dev. **1**:751–761.
18. **Muegge, K., T. M. Williams, J. Kant, M. Karin, R. Chiu, A. Schmidt, U. Siebenlist, H. Young, and S. K. Durum.** 1989. Interleukin-1 costimulatory activity on the interleukin-2 promoter via AP-1. Science **246**:249–251.
19. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
20. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5**:444–447.
21. **Quinn, J. P., N. Holbrook, and D. Levens.** 1987. Binding of a cellular protein to the gibbon ape leukemia virus enhancer. Mol. Cell. Biol. **7**:2735–2744.
22. **Schibuya, H., M. Yoneyama, and T. Taniguchi.** 1989. Involvement of a common transcription factor in the regulated expression of IL-2 and IL-2 receptor genes. Int. Immunol. **1**:43–49.
23. **Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller.** 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA **7**:47–55.
24. **Shaw, J. P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree.** 1989. Identification of a putative regulator of early T cell activation genes. Science **241**:202–205.
25. **Shevach, E. M.** 1985. The effects of cyclosporin A on the immune system. Annu. Rev. Immunol. **3**:397–423.
26. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-**

Staal, and W. C. Greene. 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. Science **238**:1575–1578.

27. **Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Sharp, and D. Baltimore.** 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. Nature (London) **323**: 640–643.
28. **Tong-Starksen, S. E., P. A. Luciw, and B. M. Peterlin.** 1989. Signalling through T lymphocyte surface proteins, TCR/CD3 and CD28, activates the HIV-1 long terminal repeat. J. Immunol. **142**:702–707.
29. **Zipfel, P. F., S. G. Irving, K. Kelly, and U. Siebenlist.** 1989. Complexity of the primary genetic response to mitogenic activation of human T cells. Mol. Cell. Biol. **9**:1041–1048.

EXHIBIT E

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo

Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5214–5218, August 1984
Immunology

Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription

(*in vitro* transcription/lymphokine gene expression/interleukin 2)

MARTIN KRÖNKE*, WARREN J. LEONARD*, JOEL M. DEPPER*, SURESH K. ARYA†, FLOSSIE WONG-STAAL†, ROBERT C. GALLO†, THOMAS A. WALDMANN*, AND WARNER C. GREENE*

*The Metabolism Branch and the †Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205

Communicated by Dewitt Stetten, Jr., April 27, 1984

ABSTRACT **Cyclosporin A (CsA) is a potent immunosuppressive agent, now gaining wide application in human organ transplantation. The immunosuppressive activity of CsA is at least in part due to inhibition of lymphokine production by activated T lymphocytes. Specifically, inhibition of T-cell growth factor (TCGF; also designated interleukin 2) production appears to be an important pathway by which CsA impairs T-cell function. To define further both the specificity of CsA and the level at which it interferes with lymphokine gene expression, we have studied its effects on TCGF mRNA accumulation as well as TCGF gene transcription. These studies were performed with a cloned human leukemic T-cell line (Jurkat, subclone 32), which can be induced with phytohemagglutinin and phorbol 12-myristate 13-acetate to produce large amounts of TCGF. In these cells, high levels of TCGF mRNA were present in induced but not in uninduced Jurkat cells as judged by hybridization to a cloned human TCGF cDNA probe. CsA completely inhibited induced TCGF mRNA accumulation at concentrations of 0.3–1.0 μg/ml, whereas low levels of appropriately sized TCGF mRNA were present at 0.01 μg/ml. In nuclear transcription experiments, CsA inhibited the synthesis of TCGF transcripts in a dose-dependent manner with complete inhibition at a concentration of 1 μg/ml. In contrast, CsA did not inhibit the expression of two other inducible genes, TCGF receptor and HT-3. Further, HLA gene expression was also less affected than TCGF in CsA-treated cells. These data suggest a relatively selective action of CsA on TCGF gene transcription.**

Cyclosporin A (CsA), a fungal metabolite, has become a valuable drug in human organ transplantation, mainly because of its ability to prevent the rejection of HLA-mismatched allografts (1–3). Although CsA has no advantage over other immunosuppressive agents with respect to the frequency of post-transplant viral infections, it is nontoxic for bone marrow stem cells and results in fewer bacterial infections (1, 4).

It has been shown that CsA acts primarily on T lymphocytes sparing the immunocompetence of B cells and macrophages (5–8). CsA immunosuppression is mediated at least in part by inhibition of lymphokine secretion by T cells, which provide requisite growth and differentiation signals for T cells, B cells, and macrophages, (9–12). Among these lymphokines is T-cell growth factor (TCGF, or interleukin 2), the principal stimulus causing proliferation of activated T lymphocytes (13). CsA acts at least in part by interfering with the production of TCGF. However, the precise mechanism by which CsA impairs T-cell function in general and TCGF production in particular remains unresolved.

In an attempt to dissect the mechanism of CsA-mediated immunosuppression, we have studied TCGF gene expression in a cloned leukemic T-cell line (Jurkat, subclone 32). Using a cloned cDNA probe for human TCGF (14), we have studied TCGF gene expression and CsA action at the level of mRNA production and gene transcription in isolated nuclei. We present evidence that CsA inhibits TCGF gene expression at the level of transcription, indicating its nuclear site of action. The inhibition of TCGF gene activation seems to be a selective feature of CsA, since the expression of HLA genes and other inducible genes, including the receptor for TCGF, was less affected.

MATERIAL AND METHODS

Cell Lines. Jurkat leukemic T cells (subclone 32) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. For induction, culture medium with 3% fetal calf serum, phytohemagglutinin (PHA, 1 μg/ml), and phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) was added to cells at a density of 4×10^6 cells per ml.

Anti-Tac Binding Assay. Monoclonal anti-Tac was tritiated to high specific activity (13.6×10^6 dpm/μg) by reductive methylation as described (15). Briefly, Jurkat cells induced in the presence or absence of CsA were suspended in RPMI 1640 medium containing 1% bovine serum albumin, human IgG (1 mg/ml, Cutter), 25 mM Hepes, and 0.1% sodium azide. Aliquots of 10^6 cells were incubated in triplicate with 4.5 ng of ^{3}H-labeled anti-Tac (^{3}H-anti-Tac) in the presence of either control antibody, RPC-5 (100 μg/ml), or unlabeled anti-Tac (100 μg/ml) for 60 min at room temperature. Thereafter, cells were centrifuged in a Microfuge through 1 M sucrose cushions to separate free and cell surface-bound ^{3}H-anti-Tac. Radioactivity of the cell pellets was measured in a liquid scintillation counter and specific ^{3}H-anti-Tac binding was determined.

Cytoplasmic Dot Blot Hybridizations. Relative levels of specific mRNA species were determined by cytoplasmic dot blot hybridization as described by White and Bancroft (16). Following induction under varying conditions, 2×10^6 Jurkat cells were lysed in a buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.5% Nonidet P-40. Nuclei were pelleted, and the supernatants were denatured in 7.4% formaldehyde/0.9 M NaCl/0.09 M sodium citrate, pH 7, by heating at 60°C for 15 min. Samples were then diluted 1:2 in 2.25 M NaCl/225 mM sodium citrate, pH 7, and spotted onto nitrocellulose filters by using a manifold filter apparatus (Schleicher & Schuell).

RNA Transfer Blotting Analysis. Total cellular RNA was prepared by a guanidine isothiocyanate method (17), and poly(A)-RNA was selected by oligo(dT)-cellulose chromatography (18). Poly(A)-RNA was size fractionated on formaldehyde-agarose gels and transferred to nitrocellulose filters (19).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CsA, cyclosporin A; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; TCGF, T-cell growth factor.

***In Vitro* Labeling of RNA in Isolated Nuclei.** *In vitro* transcription with isolated nuclei was performed according to the method of McKnight and Palmiter (20). To isolate nuclei, Jurkat cells were washed in buffer containing 10 mM Tris (pH 7.5), 2 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM dithiothreitol, and 0.3 M sucrose. Cells were lysed in the same buffer supplemented with Nonidet P-40 at a final concentration of 0.02% and centrifuged in a Beckman SW 41 rotor at 25,000 rpm over a 2 M sucrose gradient to pellet the nuclei. For *in vitro* transcription, 4–5 × 10^7 nuclei were incubated for 30 min at 26°C in a 200-μl reaction solution containing 100 mM KCl, 4.5 mM $MgCl_2$, 0.4 mM (each) ATP, CTP, and GTP, 2 mM dithiothreitol, 20% glycerol, and 0.2 mCi (1 Ci = 37 GBq) of [^{32}P]UTP (3000 mCi/mmol, Amersham). Incorporation of [^{32}P]UTP increased both with time of incubation and number of nuclei added. The reaction mix was then treated with DNase I (100 μg/ml) for 15 min at 26°C, followed by digestion with proteinase K at a concentration of 150 μg/ml. Nuclear RNA was then isolated by hot phenol/chloroform extraction and two cycles of ethanol precipitation.

cDNA Probes. The origin and characterization of the 1.1-kilobase cDNA for human TCGF has been described in detail by Clark *et al.* (14). Plasmid pBR322 DNA, containing complementary DNA specific for HLA-B7 (21), was a kind gift from S. M. Weissman (Yale University, New Haven, CT). The 1.4-kilobase insert was purified by *Pst* I digestion, preparative agarose-electrophoresis, and electroelution. HT-3 cDNA recognizes mRNA sequences that are expressed selectively in activated mature T cells and human T-cell lymphoma/leukemia virus-infected T-cell lines (22). The 1.37-kilobase HT-3 fragment, derived from a cDNA library of the HUT 102 leukemic T-cell line, was prepared as described (22). For hybridization, cDNA probes were nick-translated with [^{32}P]dCTP (3000 Ci/mmol) to specific activities of 2–5 × 10^8 cpm/μg.

Hybridization of DNA and RNA. For hybridization of RNA blots to specific cDNA sequences, nitrocellulose filters were prehybridized in 40% formamide/0.6 M NaCl/0.06 M sodium citrate, pH 7/1× concentrated Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/salmon sperm DNA (100 μg/ml)/10% dextran sulfate for 4–16 hr at 40°C. Thereafter, filters were incubated with 2–5 × 10^6 cpm of nick-translated probes per ml in the same buffer for 16 hr at 40°C. In the nuclear transcription assay, ^{32}P-labeled nuclear RNA was hybridized to an excess of specific cDNA immobilized on nitrocellulose filters in 40% formamide/0.6 M NaCl/0.06 M sodium citrate, pH 7/5 mM EDTA/0.4% $NaDodSO_4$/1× concentrated Denhardt's solution/yeast tRNA (100 μg/ml) for 72 hr at 40°C. To immobilize excess amounts of cDNA, 0.5 μg of specific sequences was denatured by boiling for 10 min and treatment with 1 M NaOH. Following neutralization, probes were subsequently spotted onto nitrocellulose filters by using a manifold apparatus from Schleicher & Schuell. Following hybridization, all filters were washed four times in 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% $NaDodSO_4$ at room temperature and then twice in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% $NaDodSO_4$ at 50°C. The filters were then exposed at −70°C to Kodak XAR film using intensifying screens. Where indicated, autoradiograms were analyzed with a Biomed SL 504 laser densitometer, and the radioactivity of nitrocellulose filters was counted in a liquid scintillation counter.

RESULTS

CsA Inhibits the Accumulation of TCGF mRNA in Induced Jurkat Cells. Previous studies have indicated that CsA inhibits TCGF production by antigen- or mitogen-activated T cells (9, 10, 12). However, these studies did not define the mechanism of CsA action. Employing a cloned cDNA probe for human TCGF, we first determined TCGF mRNA levels in cloned leukemic T cells (Jurkat, subclone 32), either uninduced or induced with PHA (1 μg/ml) and PMA (50 ng/ml) for 6 hr in the presence or absence of varying concentrations of CsA. Poly(A)-RNA was then isolated from each cell population, size fractionated on formaldehyde-agarose gels, and analyzed by RNA transfer blot hybridization with nick-translated cDNA for TCGF. As shown in Fig. 1, TCGF mRNA was readily detected as an 11–12S species in induced Jurkat cells (lane 2), whereas no TCGF mRNA was detected in uninduced cells (lane 1). CsA at concentrations of 1 μg/ml (lane 3) or 0.3 μg/ml (lane 4) completely inhibited TCGF mRNA production. As concentrations of CsA were decreased from 0.1 to 0.01 μg/ml, increasing amounts of appropriately sized TCGF mRNA were identified (lanes 5–7). As shown in lane 8, the solvent for CsA, ethanol, did not interfere with TCGF mRNA production when added at a concentration of 0.1%, which was required to prepare a final CsA concentration of 1 μg/ml. Also seen are higher molecular weight mRNA species hybridizing to the TCGF probe. These mRNAs were absent in samples from uninduced and CsA-treated cells. By using an intron probe prepared from a TCGF genomic clone (provided by G. R. Crabtree), each of these larger mRNAs hybridized while the mature TCGF mRNA did not, suggesting that each represented a precursor form of TCGF mRNA. It should be noted that in some experiments of similar design, low levels of TCGF mRNA were detected at CsA concentrations of 1.0 μg/ml.

Selectivity of CsA. CsA did not diminish cell viability or the overall synthesis of DNA, RNA, or proteins, in either unstimulated or induced Jurkat cells (data not shown). Thus, CsA did not appear to exert a general inhibitory action on cell metabolism. To further examine the possibility of selectivity of CsA inhibition, we investigated its effects on the expression of membrane receptors for TCGF. Using a monoclonal anti-human TCGF receptor antibody, anti-Tac (23, 24), we have recently demonstrated that Jurkat cells express TCGF receptors following induction with PHA and PMA (25). The results summarized in Table 1 demonstrate that

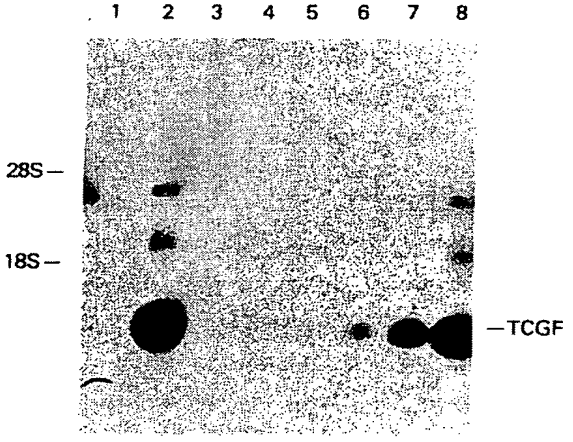


FIG. 1. Dose-dependent inhibition of TCGF mRNA accumulation by CsA. Poly(A)-RNAs from Jurkat cells were electrophoresed, transferred to nitrocellulose filters, and subsequently hybridized to nick-translated TCGF cDNA. Samples were 5 μg of poly(A)-RNA (each) of uninduced Jurkat cells (lane 1), induced with PHA (1 μg/ml) and PMA (50 ng/ml) for 6 hr (lane 2), or induced in the presence of CsA at concentrations of 1.0 μg/ml, 0.3 μg/ml, 0.1 μg/ml, 0.03 μg/ml, and 0.01 μg/ml (lanes 3–7, respectively). Lane 8 represents induction in the presence of 0.1% ethanol. The migration of 28S and 18S rRNA was determined by ethidium bromide staining.

Table 1. CsA does not inhibit PHA- and PMA-induced expression of TCGF receptors in Jurkat cells

Jurkat cells induced with	^{3}H-anti-Tac bound, pg per 10^6 cells
Medium alone	0
PHA and PMA	1114
+CsA, 1 μg/ml	
4 hr prior to induction	1506
1 hr prior to induction	1701
Simultaneously	1414
+CsA, 0.3 μg/ml	
Simultaneously	1787

Jurkat leukemic T cells were cultured for 20 hr in RPMI 1640 medium. 10% fetal calf serum with and without PHA (1 μg/ml) and PMA (50 ng/ml) and in the presence or absence of CsA. CsA was added at indicated concentrations either simultaneously with inducing agents or 1 or 4 hr prior to induction. Induced expression of TCGF receptors was measured in a radioreceptor binding assay by using ^{3}H-anti-Tac.

CsA at concentrations of 1.0 μg/ml and 0.3 μg/ml does not inhibit TCGF receptor expression in induced cells as measured by ^{3}H-anti-Tac binding. Furthermore, preincubation of cells with CsA for 4 or 1 hr prior to induction had no effect on ^{3}H-anti-Tac binding. In addition to TCGF receptor expression, we evaluated CsA effects on mRNA levels for HLA-B, constitutively expressed in Jurkat cells, and HT-3, a gene activated in Jurkat cells by PHA and PMA (22). Poly(A)-RNA from uninduced, induced, and CsA-treated cells was hybridized to nick-translated cloned cDNA probes specific for either TCGF, HLA-B7, or HT-3 (Fig. 2). As expected, induced but not uninduced Jurkat cells expressed TCGF mRNA, and this induced expression was inhibited by CsA (lanes 1–3). In contrast, similar levels of HLA mRNA were detected in uninduced and induced Jurkat cells (lanes 7 and 8). CsA only marginally decreased HLA mRNA levels (lane 9). HT-3 mRNA was not detected in uninduced Jurkat cells (lane 4). Upon induction, Jurkat cells produced HT-3 mRNA (lane 5), which was not inhibited by CsA at a concentration of 1 μg/ml (lane 6).

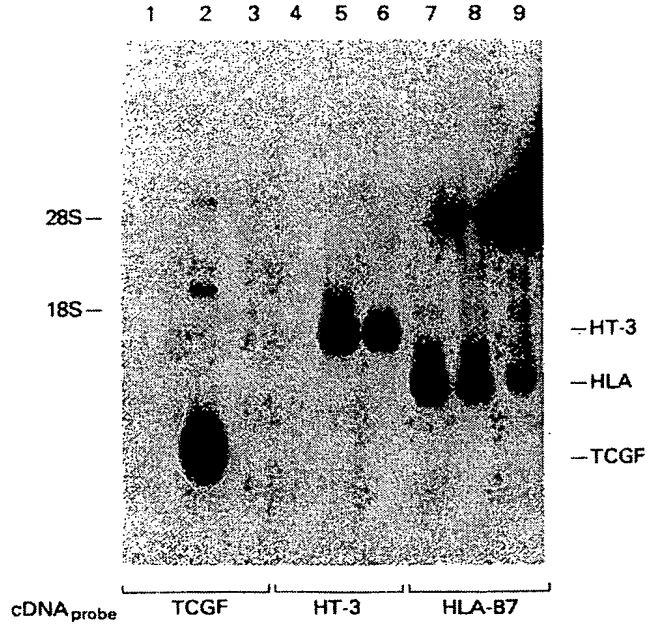


FIG. 2. Selectivity of CsA. Poly(A)-RNAs from Jurkat cells either uninduced (lanes 1, 4, and 7), induced (lanes 2, 5, and 8), or induced in the presence of CsA at 1.0 μg/ml (lanes 3, 6, and 9) were size fractionated and hybridized to [^{32}P]cDNA probes specific for TCGF (lanes 1–3), HT-3 (lanes 4–6), and HLA-B7 (lanes 7–9). The migration of 28S and 18S rRNA is indicated.

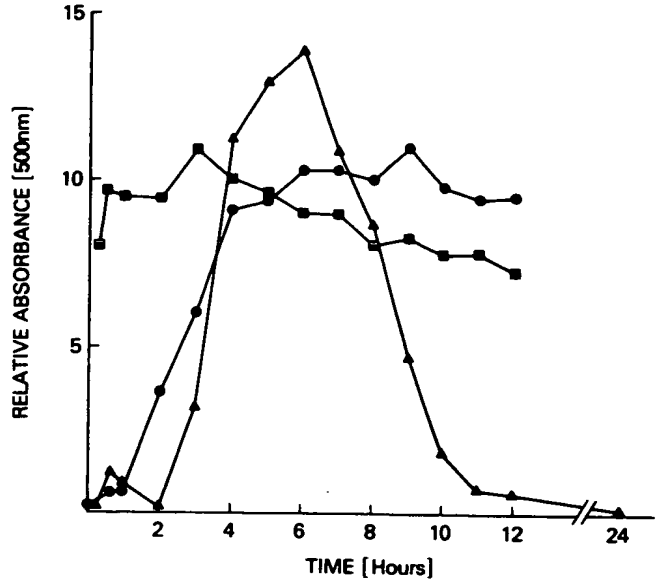


FIG. 3. Time course of TCGF, HLA, and HT-3 mRNA accumulation. Jurkat cells (2×10^6) were induced with PHA (1 μg/ml) and PMA (50 ng/ml). At the indicated times, cells were harvested, and cytoplasmic extracts were then spotted in serial 1:2 dilutions onto nitrocellulose filters and hybridized to either TCGF (▲), HLA-B7 (■), or HT-3 (●) cDNA probes. Autoradiograms were quantitated by densitometer reading and relative mRNA levels are expressed as percentage of the overall specific mRNA accumulation integrated for the time period investigated.

However, these results did not exclude the possibility of a rapid induction of HT-3 mRNA production prior to achieving effective intracellular levels of CsA. To rule out this possibility, we performed time course studies of TCGF, HT-3, and HLA mRNA accumulation employing cytoplasmic dot blot hybridizations. As shown in Fig. 3, the time course of induction was similar for both TCGF and HT-3. TCGF mRNA production was detectable at 3 hr, peaked at 6 hr, and declined to undetectable levels at 24 hr, confirming the results from a previous report (14). HT-3 mRNA accumulation started at 1 hr, peaked at 4 hr, and remained at this level for several hours. In contrast, HLA mRNA was constitutively expressed in unstimulated cells, slightly elevated at 3 hr after induction, and slowly declined over the next 9 hr.

We also determined the effects of CsA on TCGF and HT-3 mRNA levels when administered prior to, simultaneous with, or after induction with PHA and PMA. As shown in Fig. 4, 1-hr preincubation or concurrent administration of CsA resulted in almost complete inhibition of TCGF mRNA production. Thereafter, increasing amounts of mRNA levels emerged. When added 4 hr after induction, CsA did not alter TCGF mRNA levels. In contrast, CsA had no effect on HT-3 mRNA expression when added 1 hr prior to induction or at any time thereafter.

CsA Inhibits TCGF Gene Transcription. Although low levels of TCGF mRNA in CsA-treated cells were consistent with diminished TCGF gene transcription, these data did not exclude altered RNA splicing, processing, or transport combined with intranuclear RNA degradation or accelerated cytoplasmic mRNA degradation. To evaluate potential CsA effects on the transcription of TCGF-specific sequences directly, nuclei from uninduced Jurkat cells as well as from Jurkat cells induced in the presence or absence of CsA were isolated, and nascent RNA chains were allowed to elongate

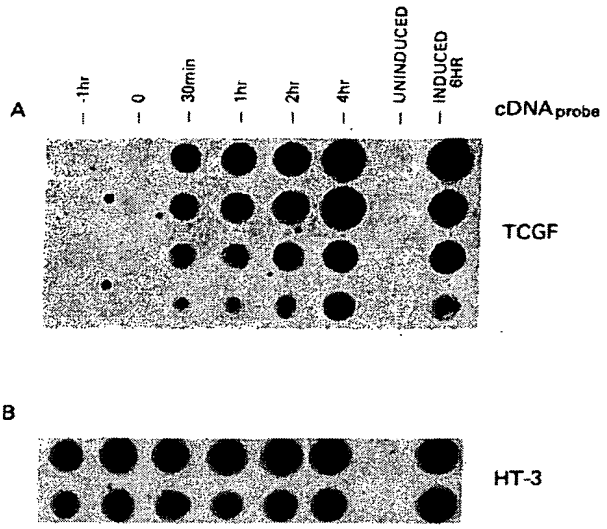


FIG. 4. Time course of CsA action. Jurkat cells were induced as described in the legend to Fig. 3 and, at the indicated times, CsA was added to a final concentration of 1 μg/ml. Serial 1:2 dilutions of cytoplasmic RNA were then spotted onto nitrocellulose filters and hybridized to either TCGF (*A*) or HT-3 (*B*) cDNA probes.

for 30 min in the presence of [^{32}P]UTP. Equivalent amounts of labeled nuclear RNA from each sample were then hybridized to excess cDNA specific for TCGF or HLA. A typical experiment is depicted in Fig. 5 and results analyzed quantitatively are given in Table 2. Significant levels of TCGF transcripts were observed in induced cells but not in uninduced cells. At a concentration of 1 μg/ml, CsA reduced TCGF transcription in nuclei from induced cells to background levels. In nuclei from cells treated with 0.1 and 0.01 μg of CsA per ml, TCGF transcription was suppressed by ≈78% and ≈67%, respectively. As expected, HLA sequences were found to be constitutively transcribed. However, in induced cells, HLA was transcribed at a 4- to 5-fold higher rate, as determined by both densitometry and counting. At 1 μg of CsA per ml, the transcription rate of HLA was intermediate between induced and uninduced cells. With decreasing concentrations of CsA, HLA transcriptional activity increased to levels observed in induced cells. As expected, nuclei from an Epstein–Barr virus-transformed B-cell line produced HLA- but not TCGF-specific transcripts. When nuclei were incubated in the presence of α-amanitin (5 μg/ml), [^{32}P]UTP incorporation was only 30–40% of untreated control, whereas the transcription of both TCGF and HLA were inhibited completely (data not shown), indicating that the transcription of both genes required intact RNA polymerase II activity.

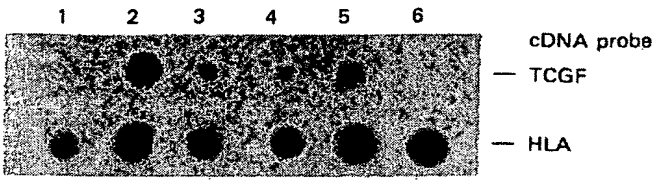


FIG. 5. CsA inhibition of TCGF gene transcription in isolated nuclei. Nuclei were isolated from unstimulated Jurkat cells (lane 1), from Jurkat cells induced for 4 hr in the absence (lane 2) or presence of CsA (1.0 μg/ml, 0.1 μg/ml, 0.01 μg/ml, lanes 3–5, respectively), and from an Epstein–Barr virus-transformed B-cell line (lane 6). Following *in vitro* transcription, RNA isolation, and hybridization to TCGF and HLA-B7 cDNA immobilized on nitrocellulose filters, hybridized ^{32}P-labeled nuclear RNA was then visualized by autoradiography.

DISCUSSION

Using a cDNA probe specific for human TCGF, we have employed RNA transfer blot analysis and *in vitro* transcriptional assays with isolated nuclei to study the effects of CsA on TCGF gene expression. We demonstrate that induction of Jurkat leukemic T cells with PHA and PMA leads to increased transcriptional activity of the TCGF gene, whereas in uninduced Jurkat cells TCGF-specific transcripts were not identified. Thus, the TCGF mRNA accumulation observed in induced cells reflects increased transcriptional activity of the TCGF gene. Similar, though slower, kinetics of TCGF mRNA accumulation in PHA-activated human lymphocytes have been reported by using an oocyte translation system (26). As noted previously (14), Jurkat TCGF mRNA levels rapidly decreased after peaking at 6 hr, suggesting a decline rate of ≈3 hr. Since TCGF transcription continues for at least 15 hr following induction (unpublished data), these data suggest that nontranscriptional regulatory mechanisms are involved in the rapid fall of TCGF mRNA levels. Since these studies were performed with a cloned T-cell line, the data indicate that the accumulation of TCGF mRNA and its subsequent decline result from intracellular events and occur in the absence of positive or negative signals from a second cell type.

It was previously reported that CsA abrogates TCGF production by activated T lymphocytes (9, 10, 12). However, it was not known whether CsA acted at the level of gene transcription, mRNA translation, or post-translational protein processing. The results of our study demonstrate that TCGF mRNA accumulation in induced Jurkat cells is diminished by CsA in a dose-dependent manner and that CsA acted by blocking TCGF mRNA transcription. Although we cannot exclude additional effects of CsA on TCGF mRNA processing, stability, or translation, these data indicate that one site of CsA action is in the nucleus at the level of TCGF gene

Table 2. Transcription of TCGF and HLA RNA in isolated nuclei of Jurkat cells

Nuclei		Input of [^{32}P]RNA, cpm × 10^{-6}	Hybridization (cpm) to		Background hybridization, cpm*	Relative transcription, ppm†	
Lane	Source		TCGF	HLA		TCGF	HLA
1	Jurkat cells uninduced	2.3	74	138	76	0.0	26.8
2	Jurkat cells induced	2.3	258	362	78	76.9	121.4
3	+ CsA, 1.0 μg/ml	2.7	88	236	84	1.4	55.4
4	+ CsA, 0.1 μg/ml	2.1	104	239	68	16.7	78.9
5	+ CsA, 0.01 μg/ml	2.5	147	448	79	26.7	148.7
6	Epstein–Barr virus-transformed B-cell line	1.9	62	574	60	1.0	263.6

Radioactivity from blots shown in Fig. 5 was measured in a liquid scintillation counter. Lanes 1–6 correspond with lanes 1–6 in Fig. 5.
*Background hybridization was determined by hybridization to pBR322.
†Background hybridization was subtracted from actual cpm and divided by input cpm to give specific hybridization values (ppm).

transcription. Inhibition of TCGF gene expression appears to be a selective feature of CsA action, since two other inducible genes investigated in this study, TCGF receptor and HT-3, were not affected by CsA. It has been suggested that CsA might fail to inhibit TCGF receptor expression because this antigen occurs at the early phase of the cell cycle preceding the expression of other activation antigens (27). However, different kinetics of expression of HT-3 and TCGF receptors could not account for their resistance to CsA treatment since (*i*) HT-3 exhibited similar kinetics of appearance compared with TCGF and (*ii*) both HT-3 and TCGF receptors were expressed in cells pretreated with CsA either 4 or 1 hr prior to the addition of inducing agents.

In addition to TCGF, CsA has been shown to inhibit interferon-γ production (11) and the expression of T-cell activation antigens such as HLA-Dr determinants (27, 28), transferrin receptors (T9, ref. 27), and T10 (27). Interestingly, TCGF regulates the expression of transferrin receptors in T cells (29), and interferon-γ enhances the expression of HLA class I and class II antigens (30, 31). Thus, CsA inhibition of TCGF gene expression could result in diminished transferrin receptor display. Similarly, CsA inhibition of the increase in HLA expression occurring after induction may be secondary to inhibition of interferon-γ production. Taken together, it is possible that CsA directly affects the expression of only a few inducible genes, while the apparent inhibition of others is the result of diminished TCGF or interferon-γ production. We believe that CsA, in addition to its clinical utility in human organ transplantation, may be valuable in further studies of differential lymphokine gene expression at the molecular level.

We thank Penny Svetlik and Nancy Peffer for excellent technical assistance. M.K. was supported by the Deutsche Forschungsgemeinschaft.

1. Borel, J. F., Feurer, C., Magnee, C. & Stähelin, H. (1977) *Immunology* **32**, 1017–1025.
2. Caine, R. Y., Rolles, K. & Thiru, S., McMaster, P., Craddock, G. N., Aziz, S., White, D. J. G., Evans, D. B., Dunn, D. C., Henderson, R. G. & Lewis, P. (1979) *Lancet* **ii**, 1033–1036.
3. Starzl, T. E., Klintmalm, G. B. G., Porter, K. A., Iwatsuki, S. & Schröter, G. P. J. (1981) *N. Engl. J. Med.* **305**, 266–269.
4. Dummer, J. S., Hardy, A., Poorsattar, A. & Ho, M. (1983) *Transplantation* **36**, 259–267.
5. Larsson, E. L. (1980) *J. Immunol.* **124**, 2828–2833.
6. Horsburgh, T., Wood, P. & Brent, L. (1980) *Nature (London)* **286**, 609–611.
7. Kunkl, A. & Klaus, G. (1980) *J. Immunol.* **125**, 2526–2531.
8. McIntosh, L. & Thomson, A. (1980) *Transplantation* **30**, 384–386.
9. Bunjes, D., Hardt, C., Röllinghoff, M. & Wagner, H. (1981) *Eur. J. Immunol.* **11**, 657–661.
10. Hess, A. D., Tutschka, P., Zhang, P. & Santos, G. W. (1982) *J. Immunol.* **128**, 360–367.
11. Reem, G. H., Cook, L. A. & Vilcek, J. (1983) *Science* **221**, 63–65.
12. Orosz, C. G., Roopenian, D. C., Widmer, M. B. & Bach, F. H. (1984) *Transplantation* **36**, 706–711.
13. Morgan, D. A., Ruscetti, F. W. & Gallo, R. C. (1976) *Science* **193**, 1007–1008.
14. Clark, S. C., Arya, S. K., Wong-Staal, F., Matsumoto-Kobayashi, M., Kay, R. M., Kaufman, R. J., Brown, E. L., Shoemaker, C., Copeland, T., Oroszlan, S., Smith, K., Samgadharan, M. G., Lindner, S. G. & Gallo, R. C. (1984) *Proc. Natl. Acad. Sci. USA* **84**, 2543–2547.
15. Depper, J. M., Leonard, W. J., Waldmann, T. A. & Greene, W. C. (1984) in *Lymphokines*, ed. Pick, E. (Academic, New York), Vol. 9, pp. 127–152.
16. White, B. A. & Bancroft, F. C. (1982) *J. Biol. Chem.* **257**, 8569–8572.
17. Glisin, V., Crkvenjakov, R. & Byus, C. (1974) *Biochemistry* **13**, 2633–2637.
18. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
19. Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, S. H. (1977) *Biochemistry* **16**, 473–477.
20. McKnight, G. S. & Palmiter, R. D. (1979) *J. Biol. Chem.* **254**, 9050–9058.
21. Sood, A. K., Pereira, D. & Weissman, S. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 616–620.
22. Manzari, V., Gallo, R. C., Franchini, G., Wetsin, E., Ceccherini-Nelli, L., Popovic, M. & Wong-Staal, F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 11–15.
23. Leonard, W. J., Depper, J. M., Uchiyama, T., Smith, K. A., Waldmann, T. A. & Greene, W. C. (1983) *Nature (London)* **300**, 267–269.
24. Robb, R. J. & Greene, W. C. (1983) *J. Exp. Med.* **158**, 1332–1337.
25. Greene, W. C., Robb, R. J., Depper, J. M., Leonard, W. J., Drogula, C., Svetlik, P., Wong-Staal, F., Gallo, R. C. & Waldmann, T. A. (1984) *J. Immunol.*, in press.
26. Efrat, S., Pilo, S. & Kaempfer, R. (1982) *Nature (London)* **297**, 236–239.
27. Miyawaki, T., Yachie, A., Ohzuki, S., Nagaoki, T. & Taniguchi, N. (1983) *J. Immunol.* **130**, 2737–2741.
28. Palacios, R. & Möller, G. (1981) *Nature (London)* **290**, 792–794.
29. Neckers, L. M. & Cossman, J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3494–3498.
30. Yoshie, O., Schmidt, H., Reddy, E. S. P., Weissman, S. H. & Lengyel, P. (1982) *J. Biol. Chem.* **257**, 13169–13172.
31. Wallach, D., Fellous, M. & Revel, M. (1982) *Nature (London)* **299**, 833–835.

EXHIBIT F

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo

0022-1767/86/1371-0150$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 137, No. 1, July 1, 1986
Printed in U.S.A.

EFFECT OF CYCLOSPORIN A AND DEXAMETHASONE ON INTERLEUKIN 2 RECEPTOR GENE EXPRESSION[1]

JOHN C. REED,[2] ASAD H. ABIDI, JAMES D. ALPERS, RICHARD G. HOOVER,[3] RICHARD J. ROBB,* AND PETER C. NOWELL[4]

*From the Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; and *E. I. DuPont de Nemours and Co., Central Research and Development Department, Glenolden, PA 19036*

Here we demonstrate that CsA and DEX, at concentrations that markedly inhibited PHA-induced proliferation and IL 2 mRNA accumulation, partially diminished the expression of receptors for IL 2 on PBMC. This inhibition of IL 2 receptor expression occurred at a pretranslational level and involved a reduction in both high affinity and low affinity forms of the receptor. Although both CsA and DEX inhibited IL 2 receptor expression by about 50%, only CsA blocked the PHA-mediated induction of IL 2 responsivity in PBMC cultures. These data provide evidence that *1*) CsA and DEX suppress the proliferation of T lymphocytes through distinct (though perhaps overlapping) mechanisms, *2*) CsA (but not DEX) blocks the PHA-mediated induction of signals necessary for T cells to become capable of proliferating in response to IL 2, and *3*) T cells regulate the expression of their genes for IL 2 and IL 2 receptors, at least in part, through independent mechanisms.

Cyclosporin A (CsA),[5] a cyclic undecapeptide and fungal metabolite, and dexamethasone (DEX), a synthetic glucocorticosteroid hormone, are potent inhibitors of T lymphocyte proliferation induced by antigens and by mitogens (1, 2). These immunosuppressive agents bind to specific intracellular receptors in target tissues and interfere with several early events of T cell activation, including the expression of the gene for interleukin 2 (IL 2) and for the proto-oncogene c-*myc* (3–5). However, the effects of CsA and of DEX on another early event of T cell activation, the expression of receptors for IL 2, are controversial. If CsA and DEX inhibit expression of the genes for c-*myc* and IL 2, but not for IL 2 receptors, then this would suggest that IL 2 receptor gene expression is independently regulated in T cells. Conversely, if CsA and DEX do reduce IL 2 receptor gene expression, then a common pathway may regulate expression of these genes associated with early events of T cell activation.

Previous investigations of CsA and of DEX have relied upon indirect methods for assessing IL 2 receptor expression such as the use of monoclonal antibodies that do not distinguish between the biologically active high affinity form and the less important low affinity form of the IL 2 receptor, or the measurement of the proliferative response of lymphocytes to IL 2 ("IL 2 responsivity"). Consequently, conflicting results have been obtained (1, 3, 6–9). To date, no report has examined the effects of CsA and of DEX on the expression of high affinity receptors for IL 2 or has studied the effects of these immunosuppressive agents at the level of IL 2 receptor gene expression. In this study, we undertook a thorough investigation of the effects of CsA and of DEX in primary cultures of human peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA) with regard to *1*) expression of high affinity receptors for IL 2, *2*) induction of Tac antigen (high and low affinity IL 2 receptor) expression, *3*) accumulation of IL 2 receptor mRNA, and 4) acquisition of "IL 2 responsivity."

MATERIALS AND METHODS

Reagents. CsA (gift of J. Borel, Sandoz Ltd., Basel, Switzerland) was prepared as a 10 mg/ml solution in ethanol and Tween 80 (80:20), was diluted to the appropriate concentration in RPMI 1640, and was stored protected from light at 4°C. DEX (Sigma Chemical Co., St. Louis, MO) was prepared as a 0.1 M solution in sterile dimethyl sulfoxide. PHA-P from Difco (Detroit, MI) was prepared according to the manufacturer's instructions. Purified recombinant IL 2 (lot LP230B) produced in *E. coli* as described (10) was a gift of Cetus Corp. (Emeryville, CA) and was supplied with an excipient control. The recombinant IL 2 (rIL 2) and excipient control were resuspended in RPMI 1640 supplemented with 2% serum. DEX, PHA, rIL 2, and excipient control were stored in small aliquots at −70°C until use.

Cell cultures. Human PBMC were isolated from freshly obtained heparinized venous blood of healthy volunteers or from Red Cross buffy coats by Ficoll-Hypaque density gradient centrifugation. Cells were resuspended at a density of 1 to 2 × 10^6 per ml in complete medium (RPMI supplemented with 10% heat-inactivated fetal calf serum from Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and mycostatin (100 U/ml), and were cultured in 5% CO_2/95% air at 37°C for various times with PHA-P (0.5 to 1.0 μg/ml), CsA (0.1 to 1.0 μg/ml), DEX (10^{-8} to 10^{-4} M), rIL2 (1 to 1000 U/ml), excipient control, or various combinations of these reagents. CsA and DEX were always added to PBMC cultures 20 to 30 min before other reagents.

Proliferation assay. Proliferation in PBMC cultures was determined essentially as described (11). Briefly, 0.2 ml of final cell suspension was added to flat-bottomed microtiter cultures and were incubated at 37°C in 5% CO_2/95% air for 3 days. Eight hours before termination of cultures, each well was pulsed with 0.5 μCi of [^{3}H]-thymidine (specific activity 6.7 Ci/mmol; New England Nuclear, Boston, MA). Data are reported as mean cpm of triplicate cultures.

Received for publication January 21, 1986.
Accepted for publication March 28, 1986.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by National Institutes of Health Grants CA12779 and CA36403.
[2] J. C. R. is a trainee in the Medical Scientist Training Program, NIH Grant GM-05T-3221.
[3] R. G. H. is a Special Fellow of the Leukemia Society of America.
[4] Address correspondence and reprint requests to: Peter C. Nowell, M.D., Department of Pathology-G3, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104.
[5] Abbreviations used in this paper: CsA, cyclosporin A; DEX, dexamethasone; PBMC, peripheral blood mononuclear cell; rIL 2 recombinant interleukin 2; PGK, phosphoglycerate kinase.

Immunofluorescence and flow cytometry. An indirect immunofluorescence assay was performed as described in detail (12) by employing the anti-Tac (IgG2a) monoclonal antibody (13). This antibody (gift of W. C. Greene) recognizes both the high affinity and low affinity forms of the human receptor for IL 2 (14). Analysis was accomplished by fluorescence-activated cell sorter (either a FACS IV, Becton-Dickinson, Paramus, NJ, or an Ortho Spectrum III, Ortho Diagnostics, Raritan, NJ) by using log-scale amplification of the fluorescence signal. Nonspecific binding of an IgG2a control antibody (R3-367) was subtracted from specific values to determine the percentage of cells staining positively with anti-Tac (percent Tac$^+$) or the change in the relative mean fluorescence intensity of Tac antigen expression on PBMC.

Radiolabeled IL 2 and radiolabeled anti-Tac binding assays. IL 2 was purified by immunoaffinity chromatography from supernatants of PHA and phorbol-myristate-acetate-treated JURKAT cells and was radioiodinated by a modified chloramine-T method (15); ^{3}H-anti-Tac antibody was a kind gift of W. C. Greene. The relative numbers of high affinity IL 2 binding sites per cell and of anti-Tac antibody binding sites per cell were measured in binding assays as described (14). The number of binding sites per viable cells and the apparent dissociation constants (K_d) were determined from the linear portion of Scatchard plots of the data by using linear regression analysis.

RNA blot analysis. Relative levels of various mRNA in PBMC were measured by RNA blot analysis exactly as described (5, 12). After size-fractionating total cellular RNA (10 μg/lane) by electrophoresis through 1% agarose gels containing 6% formaldehyde and 2 μg/dl of ethidium bromide, gels were viewed under UV irradiation to assess the integrity of the RNA and to verify that equal amounts of RNA had been loaded in all wells. After transfer of RNA to Gene Screen Plus nylon membranes (New England Nuclear), gels were restained with ethidium bromide to verify that transfer of RNA to membranes was uniform. Prehybridization, hybridization, and washes were conducted as detailed (5, 12). The radiolabeling of plasmid DNA and the origin and specificity of cDNA probes for human IL 2 receptor, human IL 2, and human phosphoglycerate kinase (generous gifts from W. Greene, G. Crabtree, and S. Orkin respectively) have been described (3, 5, 12). Relative intensities of bands on autoradiograms were quantified by scanning-laser densitometry at 600 nm (CS-930; Shimadzu, Kyoto, Japan).

RESULTS

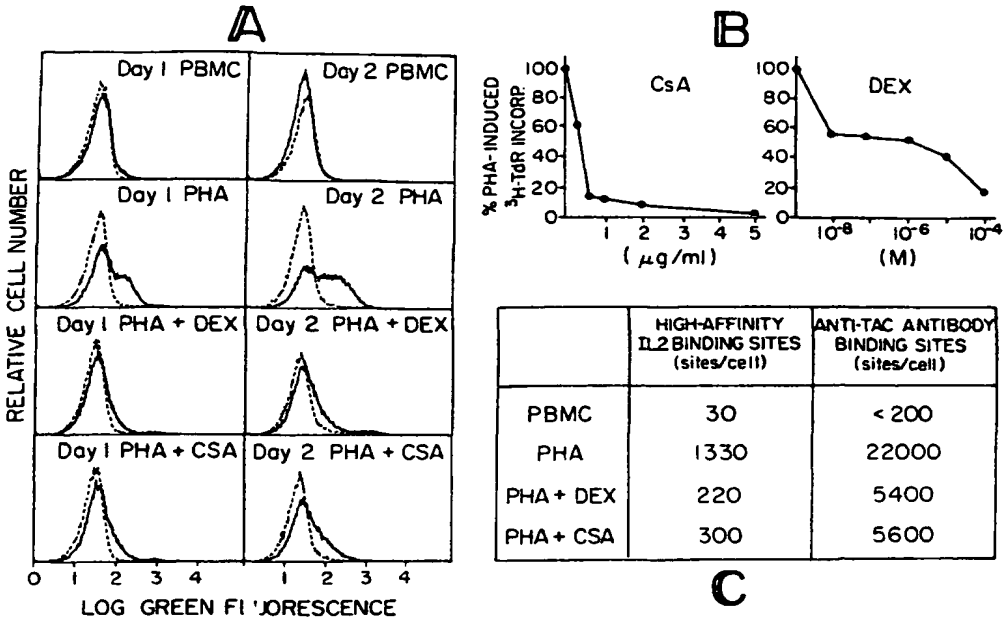
Figure 1 shows representative data from several experiments in which the inhibitory effects of CsA and of DEX on PHA-induced proliferation and IL 2 receptor expression were investigated. In Figure 1*B*, various concentrations of CsA and of DEX were added at the initiation of PHA-stimulated cultures of PBMC, and the minimal concentrations required to achieve 80 to 90% inhibition of proliferation were determined. Consistent with previous reports (1, 2, 7–9), CsA produced marked suppression of PHA-induced proliferation at concentrations ≥0.5 μg/ml. To obtain 80 to 90% inhibition of proliferation induced by 1 μg/ml PHA-P, 10^{-4} M DEX was required. Though 10^{-6} M DEX is typically used to inhibit IL 2 production (1, 4, 16), Larsson has shown that this concentration diminishes lectin-induced proliferation by only about 50% (1). In addition, Nouri and Nouri have reported that inhibition of proliferation by DEX is dependent on the concentration of PHA used to stimulate PBMC, whereas inhibition mediated by CsA is not (2). Thus, the minimal concentrations of CsA and of DEX that produced 80 to 90% inhibition of proliferation in these experiments (Fig. 1*B*) are in reasonable agreement with published data (1, 2, 6–9).

Figure 1*A* is representative of several experiments in which relative levels of expression of Tac antigen were measured (by immunofluorescence assay) on PBMC cultured for 1 or 2 days in the presence of 1 μg/ml PHA-P, 1 μg/ml CsA, 10^{-4} M DEX, or various combinations of these reagents. At these concentrations, CsA and DEX caused ≥80% inhibition of PHA-induced proliferation and diminished levels of Tac antigen expression by approximately 50%. Specifically, for seven experiments (data not shown), 1 μg/ml CsA diminished the PHA-induced increase in the percentage of PBMC staining positively with anti-Tac antibody (percent Tac$^+$) by (mean ± standard deviation) 40 ± 18% (day 1) and 45 ± 14% (day 2). DEX (10^{-4} M) reduced the percent Tac$^+$ value by 52 ± 22% (day 1) and 66 ± 18% (day 2). CsA (1 μg/ml) and DEX (10^{-4} M) also decreased the relative mean fluorescence intensity (Δ relative mean fluorescence intensity) of cells stained with anti-Tac antibody by 32 ± 12% (day 1, CsA), 52 ± 9% (day 2, CsA), 48 ± 21% (day 1, DEX), and 53 ± 21% (day 2, DEX).

Because anti-Tac antibody does not distinguish between high affinity and low affinity forms of the IL 2 receptor (14), we also determined the effects of CsA and of DEX on the PHA-induced expression of high affinity receptors for IL 2 with the use of a quantitative binding assay. Figure 1*C* compares the relative numbers of ^{125}I-IL 2 binding sites per cell (high affinity) and of ^{3}H-anti-Tac antibody binding sites per cell (high affinity and low affinity) on PBMC recovered from cultures. As shown, both 1 μg/ml CsA and 10^{-4} M DEX diminished the number of ^{125}I-IL 2 binding sites per cell induced on PBMC by PHA, but did not alter the apparent K_d of the high affinity receptor (see Fig. 1*C* and legend). The relative numbers of ^{3}H-anti-Tac binding sites per cell were also reduced to a similar extent as the ^{125}I-IL 2 binding sites, indicating that there was not a preferential decrease in either class of binding sites. The degree to which expression of high affinity IL 2 receptors was reduced varied among experiments, with 1 μg/ml CsA producing decreases of 30, 33, and 83% (mean 49%) and 10^{-4} M DEX mediating reductions of 32, 48, and 78% (mean 53%).

In an effort to delineate the mechanism whereby CsA and DEX suppress the PHA-induced expression of IL 2 receptors on PBMC, we investigated the effects of these inhibitory agents on the accumulation of mRNA for IL 2 receptors (see Fig. 2) by using standard RNA blotting techniques. As shown, 1 μg/ml CsA and 10^{-4} M DEX diminished levels of IL 2 receptor mRNA in PHA-stimulated PBMC by 53 ± 10% and 61 ± 8% respectively. For comparison, these blots were also hybridized with a ^{32}P-IL 2 cDNA probe after elution of the IL 2 receptor probe. In contrast to their partial inhibitory effects on IL 2 receptor mRNA accumulation, 1 μg/ml CsA and 10^{-4} M DEX completely blocked the PHA-induced accumulation of mRNA for IL 2 (Fig. 2). The PHA-induced elaboration of IL 2 into culture supernatants was also inhibited completely in cultures containing either CsA or DEX (not shown).

Hybridization of RNA blots with a labeled phosphoglycerate kinase (PGK) probe demonstrated the specificity of CsA- and DEX-mediated inhibition of IL 2 and IL 2 receptor mRNA accumulation. Though CsA and DEX produced reductions in the modest increase in the levels of PGK mRNA that occurs with PHA stimulation, this was not comparable with the magnitude of suppression obtained for IL 2 and IL 2 receptor mRNA. CsA and DEX did not reduce the production of this control mRNA species below its constitutive, baseline levels in resting PBMC (see Fig. 2), indicating that these immunosuppressive agents may block activation-associated changes in gene expression but do not impair gene expression in general.



	HIGH-AFFINITY IL2 BINDING SITES (sites/cell)	ANTI-TAC ANTIBODY BINDING SITES (sites/cell)
PBMC	30	< 200
PHA	1330	22000
PHA + DEX	220	5400
PHA + CSA	300	5600

Figure 1. CsA and DEX partially inhibit IL 2 receptor expression in PHA-stimulated cultures of PBMC. *A*. PBMC recovered from cultures containing 1 μg/ml PHA-P, 1 μg/ml CsA, 10^{-4} M DEX, or various combinations of these reagents were reacted with primary antibody, either anti-Tac (*solid line*) or control antibody (*dashed line*), followed by secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse antiserum), and were analyzed with a FACS IV by using log-scale amplification of the fluorescence signal. Recovery of PBMC from cultures was ≥80% of input cells, with ≥85% viability. Data shown are representative of seven experiments. *B*. Incorporation of [^{3}H]thymidine (*^{3}H-TdR*) was measured during the last 8 hr of 3 day cultures of PBMC containing 1 μg/ml PHA-P and various concentrations of CsA and of DEX. Viability of cells in all cultures was ≥85% as determined by trypan blue dye exclusion. Adding dimethyl sulfoxide or ethanol (1/1000, v/v) to cultures produced no inhibition of PHA-induced DNA synthesis. Data shown here are representative of three experiments and represent mean cpm of triplicate cultures expressed as a percentage of the PHA-induced response (100%). Standard deviations for triplicates were always ≤15% of the mean cpm. Note that DEX is displayed in log-scale. *C*. Relative numbers of ^{125}I-IL 2 and of ^{3}H-anti-Tac binding sites per viable cell were determined for PBMC recovered from cultures after 2 days. Values were calculated from Scatchard plots of the data and are based on an estimated specific activity of 1.5×10^6 dpm/pmol for ^{125}I-IL 2 and of 8.3×10^4 dpm/pmol for ^{3}H-anti-Tac antibody. The apparent K_d for ^{125}I-IL 2 binding sites was 8 to 10 pM.

Because IL 2 has been previously demonstrated to augment expression of IL 2 receptors in PHA-stimulated PBMC (5, 20), we next investigated whether purified rIL 2 could restore proliferation and IL 2 receptor expression in PBMC cultures inhibited by CsA or by DEX. Figure 3*A* illustrates the effects of various concentrations of rIL 2 added at the initiation of cultures on proliferation. As shown, rIL 2 augmented proliferation in DEX-containing PBMC cultures in a concentration-dependent manner. In CsA-inhibited cultures, in contrast, rIL 2 had little effect even when the concentration of CsA was reduced to 0.5 μg/ml (see Fig. 3*A*). Thus, consistent with a previous report (1), CsA blocked the PHA-mediated induction of IL 2 responsivity in PBMC cultures, whereas DEX did not.

The IL 2-induced restoration of proliferation in PBMC cultures that contained DEX was accompanied by an augmentation of IL 2 receptor expression as measured by increased fluorescence of cells stained with anti-Tac antibody (Fig. 3*B*) and by elevated levels of IL 2 receptor mRNA (Fig. 3*C*). The restoration of proliferation and of IL 2 receptor expression mediated by rIL 2 in DEX-containing cultures, however, was only partial and never equaled that observed in PBMC cultures stimulated with PHA plus rIL 2 (Fig. 3). Adding rIL 2 to these cultures did not elevate levels of IL 2 and PGK mRNA (not shown).

In contrast to DEX-treated cultures, rIL 2 did not restore IL 2 receptor expression in CsA-inhibited cultures (Fig. 3*B*). This finding agrees with a previous study that used murine T cells (8), but differs from a recent report that employed human PBMC (9). The culture conditions of this latter report, however, differed significantly from ours.

DISCUSSION

The data presented here demonstrate that CsA and DEX at concentrations that inhibited PHA-induced proliferation by 80 to 90% diminished by about 50% (on average) the expression of receptors for IL 2 on PBMC. This inhibition of IL 2 receptor expression occurred at least in part at a pretranslational level and involved a reduction in both high affinity and low affinity forms of the receptor. Previously, we presented evidence that IL 2 receptor expression is regulated in PHA-activated PBMC by at least two pathways: one that is IL 2 dependent, reflecting the capacity of IL 2 to upregulate levels of IL 2 receptors on activated T cells, and another that is IL 2 independent, resulting directly from the stimulation of resting T cells by PHA (5). Given our findings that CsA and DEX at the concentrations used here completely inhibited the PHA-induced accumulation of IL 2 mRNA in PBMC (Fig. 2), it seems likely that these inhibitory agents ablated the IL 2-dependent component of IL 2 receptor expression. Whether they also interfered with the IL 2-independent pathway of IL 2 receptor expression cannot be addressed from these data. Nevertheless, our findings provide additional evidence (21) that the expression of genes for IL 2 and IL 2 receptors are at least in part independently regulated in T lymphocytes.

That 1 μg/ml CsA and 10^{-4} M DEX suppressed IL 2 mRNA accumulation completely but reduced IL 2 receptor mRNA levels only by about 50% (Fig. 2) indicates that IL

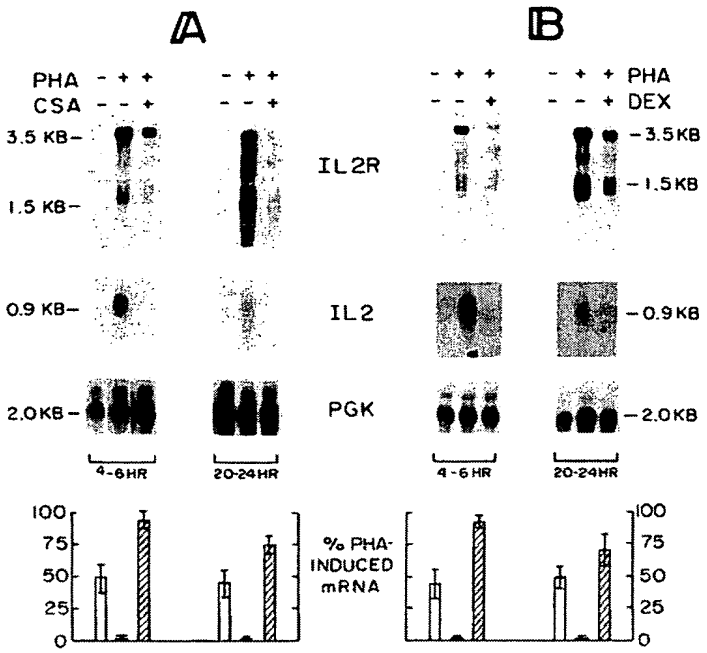


Figure 2. Effects of CsA and of DEX on accumulation of mRNAs in PBMC (representative of at least two experiments). PBMC were cultured for various times in complete medium with 1 μg/ml PHA-P, 1 μg/ml CsA, 10^{-4} M DEX, or various combinations of these reagents. Total cellular RNA was analyzed for relative levels of various mRNA by RNA blot analysis (10 μg/lane) with the use of ^{32}P-plasmids (0.5 to 1.5 × 10^9 cpm/μg) containing cDNA for IL 2 receptor (*IL2R*) (17), IL 2 (18), or PGK (19). The RNA blots used in *A* and in *B* were washed in 10 mM Tris (pH 7.4) and 0.2% SDS at 85°C for 2 hr to remove the previous probe before rehybridizing. Autoradiograms represent 1 to 3 day exposures with two intensifying screens. The sizes of the various RNA detected here were estimated from the relative positions of the 28S and 18S rRNA bands in ethidium bromide-stained gels. Scanning laser densitometry was performed on autoradiograms to quantify relative levels of mRNA for IL 2 receptors (3.5 kilobase (KB) band) □, IL 2 ■, or PGK ▨. Densitometry data are expressed as a percentage of the PHA-induced response (mean ± standard deviation for two to three experiments) for cells treated with CsA (*A*) or DEX (*B*) and are based on the relative areas under the tracings.

2 gene expression is more sensitive to inhibition by CsA and by DEX than is IL 2 receptor gene expression. In fact, at reduced concentrations, we have observed that CsA (0.1 μg/ml) and DEX (10^{-6} M) significantly reduce the levels of IL 2 mRNA in PHA-stimulated PBMC but have little effect on IL 2 receptor mRNA accumulation (unpublished). This differential sensitivity of IL 2 and IL 2 receptor genes to inhibition mediated by CsA or by DEX may explain some of the discrepancies reported by different investigators regarding the inhibitory effects of these agents (1–9). Furthermore, to inhibit very early events of T cell activation such as expression of genes for c-*myc* and IL 2 receptors, we have found it important that CsA and DEX are added to cultures 15 to 30 min before PHA is added. This latter point indicates that CsA and DEX block the PHA-mediated induction of changes in gene expression in PBMC rather than interfering with ongoing activation events, and suggests another potential explanation for some of the inconsistencies in the CsA and DEX literature.

Although both CsA and DEX reduced the levels of IL 2 receptors on PBMC on average by about 50%, only CsA blocked the induction by PHA of responsivity to IL 2 (Fig. 3). By "responsivity" we refer to the inability of CsA-inhibited cells to proliferate when exogenous IL 2 is added to cultures (Fig. 3*A*). Although we cannot exclude the possibility that some post-IL 2 binding events still occur in PBMC treated with CsA, at least two IL 2-mediated events, proliferation and IL 2 receptor upregulation, do not occur (Figs. 3*A* and *B*). Given the failure of CsA-inhibited PBMC to respond to IL 2 by proliferation and IL 2 receptor upregulation, one could argue that CsA impairs molecular events that result directly from the binding of IL 2 to its cellular receptor. This possibility seems unlikely, however, because previous studies by us (22) and by others (1, 7, 8) have demonstrated that CsA (1 to 5 μg/ml) does not interfere directly with IL 2 receptor function in that CsA fails to suppress IL 2-induced proliferation in long-term cultures of activated T cells.

What then is the basis for the observation that CsA inhibits the PHA-mediated induction of IL 2 responsivity in primary cultures of PBMC (Fig. 3) but does not interfere with IL 2 responsivity in long-term cultures of activated T cells? Clearly, the suppression of IL 2 responsivity by CsA in primary cultures is not attributable entirely to reduced IL 2 receptor levels because DEX also inhibited IL 2 receptor expression (both high and low affinity) to a similar extent as CsA (Figs. 1 and 2), yet did not block the acquisition of responsivity to IL 2 in PHA-stimulated cultures (Fig. 3). One possible interpretation consistent with the findings reported here and by others (1, 7, 8) is that CsA (but not DEX) blocks the PHA-mediated induction of other signals necessary (in addition to those signals leading to IL 2 receptor expression) for resting T cells to become capable of responding to IL 2. Once T cells have received these signals and have expressed at least low levels of IL 2 receptors, they are capable of responding to IL 2 and (as in the case of long-term cultures of IL 2-responsive T cells or preactivated T cells) are no longer susceptible to inhibition by CsA. The combined evidence from these (Figs. 1 and 3) and previous studies (1, 7, 8) of CsA therefore suggests that the expression of high affinity IL 2 receptors is insufficient for IL 2 responsivity, and that other signals are necessary. The molecular nature of these "signals" required to couple high affinity IL 2 receptors to proliferation is unknown, but among the numerous possibilities are secretion of soluble factors other than IL 2, synthesis of regulatory proteins that associate with IL 2 receptors in the membranes of T cells, expression of proto-oncogenes, and activation of intracellular protein kinases.

The suggestion that IL 2 receptor-expressing cells may require additional signals to become responsive to IL 2 is not unlike what is observed in other growth factor receptor systems. For example, fibroblasts possess high affinity receptors for epidermal growth factor but cannot respond to epidermal growth factor until rendered "competent" by prior exposure to platelet-derived growth factor or other appropriate stimuli (23). Our results are also consistent with the studies of Cantrell and Smith (24) and of Depper et al. (20) regarding the regulation of IL 2 receptor expression in long-term cultures of activated T cells. These investigators showed that despite the addition of high amounts of IL 2 to activated T cells, which might be expected to upregulate IL 2 receptor expression and to indefinitely maintain proliferation, long-term cultures of these cells instead eventually lost their responsivity to IL 2 unless periodically restimulated with PHA. Thus, PHA appears to deliver as yet undefined signals that allow activated T cells to respond to IL 2 by upregu-

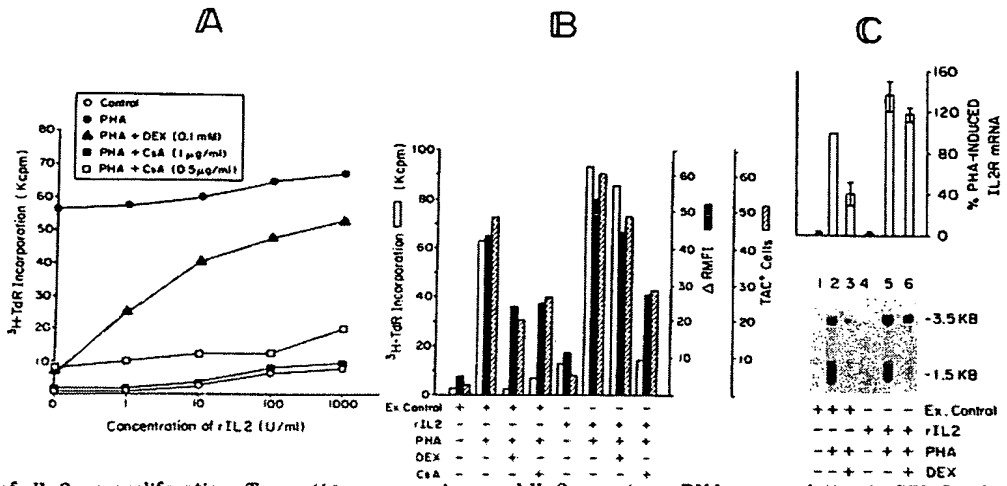


Figure 3. Effects of rIL 2 on proliferation, Tac antigen expression, and IL 2 receptor mRNA accumulation in PBMC cultures containing CsA or DEX. *A*. Incorporation of [^{3}H]thymidine (^{3}*H-TdR*) was measured during the last 8 hr of 3 day cultures of PBMC containing 1 μg/ml PHA-P, 0.5 to 1.0 μg/ml CsA, 10^{-4} M DEX, 0 to 1000 U/ml rIL 2, or various combinations of these reagents as shown in the inset. Data (representative of three experiments) are expressed as mean cpm of triplicate cultures (SD = ≤15%). *B*. Indirect immunofluorescence analysis of Tac antigen expression was performed as in Figure 1*A* by using an Ortho Spectrum III flow cytometer. Data (representative of four experiments) are expressed as the percentage of Tac$^+$ and as the change in the relative mean fluorescence intensity (Δ*RMFI*) after correcting for nonspecific binding of a control antibody R3-367. Incorporation of ^{3}H-TdR in companion microtiter cultures was assessed as in *A*. *C*. Relative levels of IL 2 receptor (*IL 2R*) mRNA were determined by RNA blot analysis as described under Figure 1. Shown are autoradiogram (representative of two experiments) and densitometric quantification of data expressed as a percentage of the PHA-induced response. Concentrations of reagents used in *B* and *C* were 1 μg/ml PHA-P, 1 μg/ml CsA, 10^{-4} M DEX, 100 U/ml rIL 2, and excipient control (*Ex. control*, used at a dilution equivalent to 100 U/ml of rIL 2) with (+) denoting presence and (−) absence in PBMC cultures.

lating their levels of IL 2 receptors and then proliferating. Whatever the nature of these signals, they must be fairly long-lived because the growth of PHA-activated T cells can be maintained for 1 to 2 weeks in medium containing IL 2 without mitogenic lectins (20, 24).

Acknowledgments. We thank W. Greene, G. Crabtree, S. Orkin, and J. Borel for reagents, A. Pickard for FACS analysis, W. Fore for photographic artwork, and L. Delpino for manuscript preparation.

REFERENCES

1. **Larsson, E. L.** 1980. Cyclosporin A and dexamethasone suppress T cell responses by selectively acting at distinct sites of the triggering process. *J. Immunol. 124:2828.*
2. **Nouri, G., and A. M. E. Nouri.** 1981. Comparison of the inhibition by glucocorticosteroids and cyclosporin A of mitogen-stimulated human lymphocyte proliferation. *Clin. Exp. Immunol. 44:287.*
3. **Krönke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldmann, and W. C. Greene.** 1984. Cyclosporin A inhibits T cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA 81:5214.*
4. **Arya, S. K., F. Wong-Staal, and R. C. Gallo.** 1984. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. *J. Immunol. 133:273.*
5. **Reed, J. C., P. C. Nowell, and R. G. Hoover.** 1985. Regulation of c-*myc* mRNA levels in normal human lymphocytes by modulators of cell proliferation. *Proc. Natl. Acad. Sci. USA 82:4221.*
6. **Bettens, F., F. Kirstensen, C. Walker, V. Schwulera, G. D. Bonnard, and A. L. de Weck.** 1984. Lymphokine regulation of activated (G1) lymphocytes. II. Glucocorticosteroid and anti-Tac-induced inhibition of human T lymphocyte proliferation. *J. Immunol. 132:261.*
7. **Miyawaki, T., A. Yachie, S. Ohzeki, T. Nagoki, and N. Taniguchi.** 1983. Cyclosporin A does not prevent expression of Tac antigen, a probable TCGF receptor molecule, on mitogen-stimulated human T cells. *J. Immunol. 130:2737.*
8. **Lillehoj, H. A., T. R. Malek, and E. M. Shevach.** 1984. Differential effect of cyclosporin A on the expression of T and B lymphocyte activation antigens. *J. Immunol. 133:244.*
9. **Solbach, W., K. Heeg, D. J. Von Steldein, M. Rollinghoff, and H. Wagner.** 1985. On the partial suppression of IL2-receptor expression and the prevention of lectin-induced lymphoblast formation by cyclosporin A. *Clin. Exp. Immunol. 60:501.*
10. **Wong, A., S. D. Lu, and D. F. Mark.** 1984. Site-specific mutagenesis of the human interleukin 2 gene: structure-function analysis of the cysteine residues. *Science 224:1431.*
11. **Reed, J. C., R. J. Robb, W. C. Greene, and P. C. Nowell.** 1985. Effect of wheat germ agglutinin on the interleukin pathway of human T lymphocyte activation. *J. Immunol. 134:314.*
12. **Reed, J. C., W. C. Greene, R. G. Hoover, and P. C. Nowell.** 1985. Monoclonal antibody OKT11A inhibits and recombinant interleukin 2 (IL 2) augments expression of IL 2 receptors at a pretranslational level. *J. Immunol. 135:2478.*
13. **Uchiyama, T., S. Broder, and T. A. Waldmann.** 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac$^+$ cells. *J. Immunol. 126:1393.*
14. **Robb, R. J., W. C. Greene, and C. M. Rusk.** 1984. Low and high affinity cellular receptors for interleukin 2: implications for the levels of Tac antigen. *J. Exp. Med. 160:1126.*
15. **Robb, R. J., P. C. Mayer, and J. Garlick.** 1985. Retention of biological activity following radioiodination of human interleukin 2: comparison with biosynthetically labeled growth factor in receptor binding assays. *J. Immunol. Methods 81:15.*
16. **Gillis, S., G. R. Crabtree, and K. A. Smith.** 1979. Glucocorticosteroid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J. Immunol. 123: 1624.*
17. **Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphrey, R. J. Robb, M. Krönke, P. B. Svetlik, N. J. Peffer, T A. Waldmann, and W. C. Greene.** 1984. Molecular cloning and expression of cDNAs for the human interleukin 2 receptor. *Nature 311:626.*
18. **Holbrook, N. J., K. A. Smith, A. J. Fornace Jr., C. M. Comlau, R. L. Wiskoch, and G. R. Crabtree.** 1984. T cell growth factor: complete nucleotide sequence and organization of the gene in normal and malignant cells. *Proc. Natl. Acad. Sci. USA 81:1634.*
19. **Michelson, A. M., A. F. Markham, and S. H Orkin.** 1983. Isolation and DNA sequence of a full-length cDNA clone for human X chromosome-encoded phosphoglycerate kinase. *Proc. Natl. Acad. Sci. USA 80:472.*
20. **Depper, J. M., W. J. Leonard, C. Drogula, M. Krönke, T. A. Waldmann, and W. C. Greene.** 1985. Interleukin 2 (IL2) augments transcription of the IL2 receptor gene. *Proc. Natl. Acad. Sci. USA 82:4230.*
21. **Greene, W. C., R. J. Robb, J. M. Depper, W. J. Leonard, C. Drogula, P. B. Svetlik, F. Wong-Staal, R. C. Gallo, and T. A. Waldmann.** 1984. Phorbol diester induces expression of Tac antigen on human acute T lymphocyte leukemic cells. *J. Immunol. 133:1042.*
22. **Reed, J. C., D. Sabath, R. G. Hoover, and M. B. Prystowsky.** 1985. Recombinant interleukin 2 regulates levels of c-*myc* mRNA in a cloned T lymphocyte. *Mol. Cell. Biol. 5:3361.*
23. **Leof, E. B., W. Wharton, J. J. Van Wyck, and W. J. Pledger.** 1982. Epidermal growth factor (EGF) and somatomedin C regulate G1 progression in competent BALB/c-3T3 cells. *Exp. Cell Res. 141:107.*
24. **Cantrell, D. A., and K. A. Smith.** 1983. Transient expression of interleukin 2 receptors (consequences for T cell growth). *J. Exp. Med. 158:1895.*

EXHIBIT G

of Response to June 26, 2008 Final Office Action, Petition for Three-Month Extension of Time, and Supplemental Information Disclosure Statement

Applicants: David Baltimore, et al.
Serial No.: 10/037,341
Filed: January 4, 2002
Group Art Unit: 1636
Examiner: D. Guzo